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THE THYROTROPIN RECEPTOR IN GRAVES' DISEASE

by

(C) MARIA de los ANGELES GARCIA AMEIJEIRAS

A THESIS

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Date....12 August 1983.....

ABSTRACT

The human thyroid thyrotropin receptor (TSH-R) has been partially purified and its interaction with immunoglobulins from patients with Graves' disease (Graves' IgG) has been investigated.

TSH affinity chromatography of the solubilized thyroid membrane proteins yielded a TSH-R preparation purified by approximately 140. Contaminants such as thyroglobulin were still present in concentrations 3000 times lower than in the original solubilized membrane preparation. Characterization of the TSH-R revealed a sedimentation coefficient between 4.2 and 4.9 S. The TSH-R - ^{125}I-TSH complex had a mobility similar to aldolase in both sucrose gradients and in polyacrylamide gel electrophoresis. Further purification of the TSH-R by Graves' IgG immunoabsorbents was attempted without improvement of the results obtained by TSH affinity chromatography above. Monoclonal antibodies raised against solubilized thyroid membrane proteins, did not allow further purification of the TSH-R.

Initial studies demonstrated that the thyrotropin binding inhibition effect (TBI) of a given Graves' IgG varied depending on the TSH-R preparation procedure. That is, correlation was found between the TBI values in crude and purified TSH-R preparations made from the same gland.

The binding of ^{125}I-TSH to human thyroid TSH-R preparations was enhanced by the presence of n-acetyl-neuraminic acid (NANA) but not by other carbohydrates tested. TBI effect of Graves' and normal IgG was more marked in the presence of NANA, but the increment was parallel and not significantly different between the two groups.

Some Graves' IgGs at very low concentrations can produce an increase in ^{125}I-TSH binding to TSH-R. This effect was found to be species specific since it was observed on TSH-R preparations from human thyroid, and was less marked in bovine thyroid. However, this effect was absent in guinea pig fat cell membranes or porcine thyroid preparations.

Cholera toxin (CT), Graves' IgG and TSH can stimulate the production of cyclic AMP (cAMP) in thyroid cells. Labelled CT specifically binds to particulate and solubilized thyroid and guinea pig fat cell membrane proteins, but the binding is not influenced by the presence of TSH. Conversely, unlabelled CT did not significantly affect ^{125}I-TSH binding to the membranes.

Rat thyroid cells in culture (FRTL cells) were used for identification of the IgG binding sites. Membrane proteins were phosphorylated (^{32}P) in the presence or absence of TSH, solubilized and precipitated with Graves' or normal IgG. Electrophoretic analysis of the precipitates showed that two protein bands with molecular weights of 144,000 and 132,000, were precipitable by Graves' IgG and to a much lesser degree with normal IgG. Membrane proteins phosphorylation was independent of the presence of TSH.

Identification of human thyroid TSH-R among electrophoretically separated membrane proteins transferred to nitrocellulose (NC) paper was done by binding of ^{125}I-TSH. Labelled TSH specifically bound to two proteins with molecular weights of 128,000 and 80,000. Binding was not inhibited by excess LH, HCG, prolactin, CT or Graves' IgG. Binding sites for Graves' IgG in the same system were detected by labelling with ^{125}I-goat-antihuman IgG. Multiple binding sites were

observed, none of them having the same molecular weight as the ^{125}I-TSH binding proteins.

In conclusion, partial purification of TSH-R was achieved by TSH affinity chromatography and the TSH-R appeared to be comprised of two molecules with molecular weights of 128,000 and 80,000. Cholera toxin stimulates the thyroid cell through a different receptor than TSH, and Graves' IgG does not seem to bind to the same binding site as TSH.

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ITACA

Cuando comiences el viaje a Itaca,
ruega para que el camino sea largo,
lleno de aventuras y descubrimientos.

Ruega que el camino sea largo,
que haya muchas madrugadas,
que entres en puertos que tus ojos ignoraban,
que vayas a ciudades a aprender de los que saben.

Guarda siempre en tu corazon la idea de Itaca.

Tienes que llegar. Es tu destino,
pero no fuerzes la travesia,
es preferible que tardes muchos anos,
que seas viejo cuando fondees la isla,
rico de todo lo que habras ganado haciendo el camino,
sin esperar que te de mas riquezas.

Itaca te ha dado el bello viaje,
sin ella no habrias salido.

Y si la encuentras pobre, no es que Itaca
te haya enganado. Sabio como te has hecho
sabras lo que quieren decir los Itacas . .

Kavafis

When you start the way to Itaca
wish it to be a very long way
full of adventures and discoveries.

Wish to way to be very long,
that there be many sunrises and
when you will go into harbors that your eyes had previously ignored
that you will be in towns learning from those who know.

Keep always within you, the idea of Itaca
you must get there, it's your destiny
But do not hurry a Fate
rather take many years on it

so you will be old when arriving at the end
rid of everything you have won by taking the way,
not expecting more treasures from it.

Itaca gave you the beautiful trip
you would not have started without it
and if you find it poor, it is not that Itaca has lied to you
Wise as you became you will know what the Itaca means.

CHAPTER I

GENERAL INTRODUCTION

Graves' disease is characterized by hyperfunction of the thyroid gland under the autonomous stimulation of circulating immunoglobulins. Physiologically, thyroid function is principally controlled by the action of thyrotropin (TSH), which is secreted by the anterior pituitary under the regulation of thyrotropin releasing factor (TRH) and the active thyroid hormones, tetraiodothyronine (T_4) and triiodothyronine (T_3). TSH stimulates the thyroid cell by binding to its specific receptor (TSH-R), thus activating the cell membrane enzymes (mainly adenylate-cyclase) responsible for the production of second messengers, which stimulate the intracellular mechanisms responsible for the ultimate production and secretion of T_3 and T_4.

Until 1956 it was believed that the thyroid hyperfunction of Graves' disease was due to increased amounts or activity of the stimulating hormone TSH. It was while investigating a bioassay for TSH that Adams and Purves (1956) discovered that the sera of patients with this disease produced a somewhat different stimulation of the guinea pig thyroid than did the sera from normal subjects. The difference was a prolonged action, which reached a peak later than expected. The discovery provided evidence for the existence in these patients of a substance different from TSH, which was designated the long-acting thyroid stimulator (LATS) (Adams 1961 and Adams et al. 1961). The new bioassay, consisting of the measurement of ^{131}I uptake by the thyroid gland, was modified by McKenzie (1958) to be used in mice, thus allow-

ing the testing of multiple samples, and is currently known as the McKenzie bioassay.

In further studies of the LATS substance, it was found that its prolonged and stronger activity with respect to TSH, was due to the longer half life of the former (McKenzie 1961, Adams 1960), thus reinforcing the already strong suspicion that the two substances were different compounds.

The origin of the LATS was the main focus of research during the subsequent years, resulting in its identification as an immunoglobulin G by Meek et al. (1964) and Kriss et al. (1964). Graves' disease was thus recognized as an autoimmune disorder. Although derangement of self-tolerance had been previously observed in thyroid pathology (Doniach et al. 1957, Witebsky et al. 1957), the identification of LATS constituted the first description of an antibody as a stimulator of an endocrine cell function.

However, since it was observed that a large proportion of patients with severe Graves' disease did not present with detectable LATS activity, the possibility of an observing factor in the sera of LATS-negative patients was investigated by Adams et al. They were able to find a serum component that prevented the LATS activity, which could be neutralized by thyroid tissue [a finding previously described by Kriss et al. (1964)], among the LATS negative sera (Adams et al. 1967). This substance was designated LATS-Protector (LATS-P), and was also found to be an immunoglobulin which did not react with the mouse thyroid (Adams et al. 1971), but stimulated the thyroid of normal human volunteers (Adams et al. 1974).

An effort was made to devise an assay for the detection and measurement of Graves' immunoglobulins devoid of the complications of the described bio-assays, and that would be sensitive enough to detect the thyroid stimulating immunoglobulin (TSI) in the majority, if not all patients with Graves' disease. Several authors described different assays based on the various functions seen in the thyroid cell when stimulated with TSH (Yamashita et al. 1972, Onaya et al .1973, McKenzie et al. 1976, Kendall-Taylor et al. 1980). Among these, the cyclic AMP stimulation assay of McKenzie et al. (1976) and its variations are currently being used in many laboratories.

Of the possible thyroid cell binding sites for the immunoglobulins from Graves' disease patients (Graves' IgG) the TSH-R was a prime candidate, since the IgGs appeared to exert the same end effect as TSH, though with the previously discussed differences. Mehdi et al. (1973) and Smith et al. (1974) were the first to obtain a more direct indication that the Graves' IgG were antibodies to the TSH-R, and developed an assay for the detection of such antibodies by measuring their TSH .binding inhibition activity (TBI assay).

Earlier studies by Edmonds et al. (1970) showed that lymphocytes from Graves' patients appeared to cause a slight stimulation of thyroid cell ^{125}I uptake due to the presence of surface IgG. Knox et al. 1976a and 1976b, demonstrated that cultured peripheral lymphocytes of patients with Graves' disease could be stimulated by normal human thyroid tissue homogenates or by phytohemaglutinin (PHA) alone. The cultured lymphocytes produced antibodies that were able to stimulate cAMP production in human thyroid slices. It was apparent from these studies that no antigenic site alteration was necessary for the induc-

tion of thyroid stimulating antibody (TSAb) production since it was only the lymphocytes from Graves' patients, and not normal controls that were stimulated by normal thyroid tissue, suggesting that the triggering factor in the development of Graves' disease was a failure of the immunological surveillence and not an alteration of the TSH-R that would render it immunogenic. However, studies by von Westarp et al. (1977) demonstrated that Graves' tissue was immunogenically different in that an antibody raised against one of its fractions that contained the TSH-R did not react with thyroid preparations from non-toxic goiter or Hashimoto's thyroiditis, but competed with both LATS positive sera and TSH for the TSH-R of Graves' disease thyroid tissue.

To the present time, autoantibodies to cell membrane receptors have been described in five human diseases, namely Graves' disease, myasthenia gravis (Almond et al. 1974, Drachman 1978 and Albuquerque et al. 1976), acanthosis nigricans with insulin resistance, type B (Kahn et al. 1976 and Flier et al. 1975), ataxia telangiectasia (Bar et al. 1978, Harrison et al. 1979 and Muggeo et al. 1979) and in some cases of allergic rhinitis and asthma (Venter et al. 1980). Although under some experimental conditions autoantibodies to the insulin receptors can mimic insulin action in vitro (Kasuga et al. 1978) and in some Graves' IgG exerts an unique action of in vivo stimulation of the target cell.

The presence of autoantibodies in the circulation is the result of alterations of the self-tolerance mechanisms of the immunological system (Talal et al. 1980). It was once believed that tolerance was due to deletion of immunocompetent cells, either due to disappearance

of these cells or to irreversible inhibition of their cell surface receptor for antigen. The theory, developed by Burnet (1959), explained the existence of autoimmunity as a result of somatic mutation of lymphocytes, cross-reactivity of antibodies to external antigens with self-antigens, exposure of antigens previously hidden, changes in the nature of autoantigen and/or intense stimulation of lymphocyte proliferation. The theory, presently known as the "clonal abortion" theory is mostly concerned with B lymphocytes and is supported by some studies of B cell differentiation. The regulation of B cells however, is not only dependent on the antigen presentation, but on both T and B cells sensitization. A disparity among the T lymphocyte subpopulations may trigger the differentiation of a specific B cell clone to an auto-antibody producing plasma cell, thus starting an autoimmune process. The disequilibrium of T cells might, theoretically, be due to an excess activity of helper T lymphocytes or to inhibition of suppressor T cells.

Studies of suppressor T cell number and activity in Graves' and Hashimoto's diseases have yielded conflicting results. Normal number of suppressor T cells (Canonica et al. 1981) as well as decreased total suppressor cells (Sridama et al. 1982) and function (Okita et al. 1981a) have been reported. However, these studies lack antigenic specificity resulting in the measurement of non-specific cells.

Evidence for a cell-mediated immunity in the pathogenesis of Graves' disease has been shown by Okita et al. (1980 and 1981b) who were able to demonstrate T-lymphocyte sensitization in Graves' and Hashimoto's diseases by measuring the patients lymphocytes' migration

inhibition and of lymphocytes of normal controls incubated in the presence of lymphocytes of Graves' patients.

Although it has been suggested that Graves' IgG stimulate the thyroid cell through binding to the TSH-R and that they appear in circulation as a result of alterations of the immunological surveillance mechanisms, this hypothesis has not been proven yet. To demonstrate IgG binding to TSH-R and any associated abnormalities in the immune system, purification of the TSH-R is necessary. The isolation and characterization of the TSH-R would allow comparative analytical studies between the TSH-R from normal human thyroid glands and from thyroids from patients with Graves' disease. Moreover, purified TSH-R preparations could be used for further studies of the Graves' IgG interaction with the thyroid cell, as well as for specific antigenic stimulation in the immunological studies.

However, up to the present time, a pure preparation of the TSH-R has not been obtained since antigenic contaminants such as thyroglobulin are still present in the purified preparations. This prompted further investigation into the role of the TSH-R in Graves' disease by studying the interaction of the TSH-R preparations from different sources with Graves' IgG and other possible thyroid stimulators. Finally, the identification of the TSH-R by localization of the molecule in electrophoretically separated proteins was studied, and the Graves' IgG effect on the TSH binding to the isolated receptor was compared with the results observed in the above systems.

CHAPTER II

METHODS

1. TISSUE PREPARATION

a) Thyroid Membrane Preparation

Normal human thyroid tissue was obtained at autopsy and occasionally surgically removed thyroid tissue was used. Thyroids from patients with Graves' disease were always obtained at surgery. The tissue was kept frozen at -20 or -70^{o}C until used.

Two types of thyroid membrane preparations were made, referred to as crude membrane (CM) and purified membrane (PM) and made by modifications of the methods of Smith et al. (1974) and Medhi et al. (1975) respectively. Frozen tissue was cut, with a surgical blade in thin slices and then chopped with a multiblade slicer. For CM preparation, the tissue was suspended in 0.3 M glucose pH 7.4 or 20 mM Tris HCl pH 7.4 and Polytron (PT-10 Brinkman) homogenized for a total of 30 to 60 sec at intervals of 5-10 sec with the Polytron set at speed 7-9. The suspension was filtered through 4 and 8 layers of surgical gauze and diluted to double volume with 25 mM Tris HCl pH 7.4 and centrifuged at 800 x g for 20 min in a Sorval centrifuge at 4^{o}C. After removing the top fat layer, the supernatants were collected, mixed, diluted 2:1 in the above buffer, and centrifuged at 10,000 x g for 20 min. The final pellet was suspended in the Tris solution and kept frozen until used

unless it was immediately solubilized. The procedure was carried out at 4°C keeping the material on ice.

The purified membranes were prepared by suspending the homogenized tissue in 0.01 M sodium phosphate buffer containing 0.01 M $MgCl_2$ and 0.25 M sucrose at pH 7.4 (buffer #1) and centrifuged at 800 x g for 20 min. Supernatants were pooled and centrifuged at 10,000 x g for 10 min, the pellets were suspended in the same buffer, homogenized with a Dounce glass homogenizer using 5 to 7 hand strokes and centrifuged under the same conditions. The procedure was repeated once more and all supernatants were pooled and re-centrifuged at the same speed to remove any remaining debris. The final supernatant was layered over a sucrose gradient of 2.3 M sucrose with the membranes in the phosphate - $MgCl_2$ buffer, and centrifuged at 69,000 x g for 4 h in a Beckman SW 25 rotor. The interphase was harvested and then diluted in buffer #1 and centrifuged at the same speed for 1 h. The pellets were washed twice in 100 mM Tris HCl, 50 mM $MgCl_2$, pH 7.2 and finally resuspended in the same solution.

Bovine and porcine thyroid membranes (2 months and 2 years old pig) were prepared as the CM of human thyroid.

b) Solubilization of Membrane Proteins

Thyroid membrane proteins, including the TSH receptor were solubilized by a previously described method (Cuatrecasas 1972) with Triton X-100, Triton N-101 or sodium deoxyclolate (SDOC) detergents. The procedure was the same regardless of the detergent used. Membrane suspensions (CM) were mixed with detergent (1% final concentration)

and gently stirred for 1 h at 4°C, followed by centrifugation at 200,000 x g for 2 h at 4°C. Supernatants were collected, aliquoted and kept frozen at -20°C or -70°C until used, unless the excess detergent was removed immediately.

c) Guinea Pig Fat Cell Membrane Preparation

Guinea pigs were sacrificed by ether inhalation and fat tissue from the peritoneal cavity and inguinal areas was removed. Membranes were prepared by the same method used for thyroid membrane and solubilized in Triton N-101.

d) Detergent Removal

Excess detergent was removed by Bio-beads resin (Bio-Rad) columns. Triton X-100 was removed by the method of Holloway (1973) using 5 ml bed volume Bio-beads SM-2 columns (Bio-Rad). Three mls of solubilized material were placed on top of the column and eluted at a constant rate of 3.5 ml/h, giving a final Triton X-100 concentration in the final sample of 0.03 to 0.06%. Standardization of the procedure was done using ^{3}H Triton X-100. Following the removal of the Triton X-100, the columns were washed with 25 mM Tris HCl, pH 7.4 and then could be re-used at least 3 times obtaining the same results. The procedure was done at room temperature.

Triton N-101 was removed by Amberlite XAD-2 resin (Cheetam 1979). Samples were run at variable speeds depending on the column and sample volumes. In a typical experiment, 10-12 ml of solubilized material

were run through a 30 ml bed volume column in a total time of 35 to 45 min. The procedure was done at 4°C.

SDOC was removed by dialysis at 4°C for 24 h against 100 volumes of 25 mM Tris, 0.05% SDOC, pH 7.2, with at least 2 buffer changes (Furth, 1980).

After the excess detergent was removed, samples were aliquoted and frozen at -20°C or -70°C.

e) Protein Determination

Protein concentrations were determined by the Bradford method (1976) using a Bio-Rad commercial kit and BSA as the protein for the standard curve, diluted in the same solution as the samples. One hundred microliters of diluted standard protein or sample were aliquoted in duplicate, followed by 5 ml of a 1:5 dilution of stock Bio-Rad dye previously filtered through a Whatman #1 filter paper. Samples were incubated for 5 to 15 min at room temperature and their absorbance read at 595 nm wave length. In some experiments 0.1 ml of 1 N NaOH was added to each tube and samples were heated in a 80°C water bath for 30 min prior to the addition of 3 ml of the diluted Bradford dye (Bio-Rad).

When the samples were solubilized, detergent concentrations were determined before the protein concentration determination and the same detergent concentration was kept constant for the standard curve and the samples. In those instances, the 1 N NaOH was not added.

In some cases, protein concentrations were determined by the Lowry et al. method (1951) and by the Dulley et al. (1975) modification of the same method for the solubilized samples.

Calculations of the samples protein concentration were done following linear regression analysis of the data from the standard curve.

f) Detergent Concentration Determination

The procedure used is a modification of the method described by Garewal (1973).

One hundred microliter aliquots of solubilized samples in Triton X-100 or Triton N-101, were mixed with 0.2 ml of 70% ethanol and 0.4 ml of ammonium cobaltothiocyanate reagent (2.3 M ammonium thiocyanate and 96 mM cobalt nitrate hexahydrate) and incubated for 5 min at room temperature. A 1.5 ml volume of ethylene dichloride was added to each tube and vortexed vigorously for at least 30 sec before the tubes were centrifuged at 1,500 xg for 20 min at $4^{o}C$. The blue upper phase was discarded and the absorbance of the lower phase read at 622 and 687 nm. Sample absorbance was calculated by subtracting the reading obtained at 687 nm from the value at 622 nm.

Standard curve values were obtained simultaneously using a constant BSA concentration in different known concentrations of detergent. A linear regression analysis was used to calculate the detergent concentration.

2. AFFINITY CHROMATOGRAPHY PURIFICATION OF THE TSH RECEPTOR

The solubilized TSH receptor was purified by TSH affinity chromatography on Affi-gel 10 (Bio-Rad) agarose, linked to commercially available b-TSH (Thytropar), by the method described by Cuatrecases et al. (1971).

a) Preparation of the bTSH - Affinity Chromatography Columns

Bovine TSH (bTSH) was covalently coupled to the Affi-gel 10 agarose (a N-hydroxysuccinamide ester) following the manufacturer's (Bio-Rad) instructions. Usually, 12.5 ml of wet gel (equivalent to 0.5 g of dry material) were coupled to 10 ml of bTSH (25 mg/ml). The b-TSH was dissolved in 0.1 M sodium bicarbonate buffer pH 8 and dialyzed for 16 h against the same buffer at $4^{o}C$. As a tracer ^{125}I - TSH (50,000 cpm, S.A. 50-80 µCi/µg) was added to the non-labelled bTSH to calculate the coupling efficiency.

The gel was washed under gentle suction in a sintered glass funnel, at $4^{o}C$ with 50 ml of distilled water followed by two bed volumes of the above buffer, The gel slurry was transferred to a weighing boat, weighed and mixed with the appropriate amount of ligand within 10 min. The gel was not allowed to stand at room temperature for longer than 5 min prior to the coupling reaction. The minimal amount of ligand mixed with the gel was 0.7 ml/g of moist gel. The coupling reaction proceeded for 16 h at $4^{o}C$ under continuous gentle shaking. To block the unoccupied binding sites, 0.1 ml ethanolamine/g

of gel was added and the reaction continued for 1 h at room temperature.

Columns were prepared with the coupled gel and extensively washed with 25 mM Tris, 0.05% detergent (Triton X-100 or SDOC), pH 7.2, followed by at least 3 bed volumes of 3 M NaCl in 25 mM Tris HCl and equilibration with the Tris-detergent solution.

The washes were collected and radioactivity measured in order to calculate the coupling efficiency.

Because the amount of radioactivity found in the washes was very low (close to background), one experiment was done in which a large amount of ^{125}I-TSH was included with the ligand solution.

b) Purification of the TSH-receptor

Solubilized receptor sample (in 25 mM Tris, Triton X-100 or SDOC) was applied to the top of the affinity gel. The first gel bed volume was discarded, but subsequent eluates were collected and re-run through the gel at least 6 times. For each bed volume, the column flow was stopped so as to allow the sample to be in contact with the gel for at least 30 min. Thus the total time of sample in contact with the gel was 8 to 12 h at 4^{o}C.

Non-bound material was eluted with 25 mM Tris, 0.05% detergent, until no protein could be detected. Absorbed material was eluted with 3 M NaCl in the above solution. If SDOC was the detergent used then this was avoided as 3 M NaCl created a precipitate. Columns were washed with Tris-detergent solution and kept at 4^{o}C until further use. All eluates were collected. The first peak of protein was concen-

trated in Minicon concentrators, whereas the second peak, eluted with 3 M NaCl was dialyzed against 25 mM Tris 0.05%, detergent (1:100) for 24 h, with 2 or 3 changes of buffer, followed by sample concentration, under vacuum, to the desired volume.

All procedures were done at 4^{o}C and samples were immediately frozen and stored at -20^{o} or -70^{o}C until used.

c) Electrophoresis

Electrophoretic analysis of different TSH receptor preparations was performed in 7.5% polyacrylamide disc gels (Biophore, Bio-Rad) and in 10% SDS polyacrylamide slab gels, in a Bio-Rad electrophoresis unit.

Gels were run, previous to the sample application, for 30 min in basic buffer containing glycine and Tris base with or without SDS, pH 8.3. For the disc gels, samples were electrophoresed at 140 V for 6h, stained with Coomassie blue and de-stained with standard methanol-acetic acid solution. Some gels were cut into 2 mm fractions for radioactivity determination. Solubilized TSH receptor-^{125}I-TSH complex was analyzed in these gels.

Discontinuous slab gel electrophoresis was done by the Laemmli (1970) method. Lower gels were made with 3.75 ml of lower stock gel (1.5 M Tris base, 0.4% SDS pH 8.8), 5 ml of acryl-bis solution (10% acrylamide, 0.8% bis-acrylamide), 6.25 ml of d-d H_2O, 100 µl of 10% ammonium persulphate and 5 µl of TEMED. After polymerization was achieved the upper gel solution was poured. The upper gels were made of 1 ml upper gel stock solution (0.5 M Tris base, 0.4% SDS, pH 6.8),

0.6 ml of acryl bis solution, 2.4 ml of d-d H_2O, 50 µl of 10% ammonium persulphate and 4 µl of TEMED.

Samples were dissolved in sample buffer, containing 10% glycerol, 5% mercaptoethanol (2-ME), 2% SDS, 62.5 mM Tris, and bromophenol blue, pH 6.8, and boiled for 2 min. The 0.75 mm thick slab gels were run in basic buffer, usually at 200V, 20 mA per gel for 3-4 h, followed by staining with Coomassie blue or a Silver stain (Silver-stain, Bio-Rad) as described by the manufacturers with few modifications involving mainly additional washes with d-d H_2O after the incubation with the oxidizer (0.0034 M potassium dichromate, 0.0032 N nitric acid) to reduce the background staining (Merril et al. 1979).

Slab gels were heat dried under vacuum for up to 2 h. (Bio-Rad, slab gel drier). Some gels were then used for autoradiographic analysis.

d) Continuous Sucrose Gradients

Continuous Sucrose gradients (8-25% w/v) were made in the presence or absence of 0.03% of the detergent in which the sample to be evaluated was solubilized. The gradients were equilibrated for 6h at 4°C and then the samples were carefully applied to the top of the gradients and centrifuged for 14 h at 130,000 x g in a SW60 Ti swinging bucket rotor. Gradients were in a 4 ml volume and samples did not exceed 125 µl in volume. Standard proteins were always run in parallel gradients. Gradients were fractionated at a constant speed using a multichannel pump. Fractions were tested for binding capacity to

^{125}I-TSH, or for determination of radioactivity in the samples when ^{125}I-TSH was included.

e) Thyroglobulin Assay

Measurements of thyroglobulin (Tg) concentration were determined by a modification of the method of Van Herle et a.l (1973). Tg antiserum was raised in New Zealand white rabbits using normal post-mortem human thyroid tissue as an antigen (final assay antiserum titre was 1: 90,000).

Tg was iodinated by the lactoperoxidase method in enzymo-beads as described by Karonen et al. (1975) to a specific activity of 0.56-2.25 µCi/µg.

The procedure is a regularly used research assay and was kindly performed by the staff of the Endocrine Division's research laboratory for the purpose of this study.

f) Immunoabsorbent Columns

i. Column Preparation

Human IgG or monoclonal antibodies obtained in the mouse - mouse fusions were bound to cyanogen bromide-activated Sepharose 4B agarose (CnBr Sepharose 4B gel) from Pharmacia, as described by Eveleigh et al. (1977) and Secher et al. (1980). The agarose was swollen in 1 mM HCl and washed at room temperature for 20 min with HCl followed by 0.1 M Na bicarbonate, 1 M NaCl, pH 8.3 (buffer B). IgG solutions were dialysed against buffer B prior

to the coupling reaction. The coupling reaction buffers were: 0.1 M acetate, 1 M NaCl, pH 4.0 (buffer A), 0.1 M borate, 1 M NaCl, pH 8.3 (buffer B) and 0.1 M Tris HCl, 0.1 M NaCl, pH 7.5 (solution C). Alternately, and for some of the monoclonal antibodies coupling reactions, 0.2 M bicarbonate buffer containing 0.5 M NaCl, pH 8.5 was used instead of buffer B.

Washed agarose was allowed to couple with IgG (usually 10 mg of purified IgG per g of agarose) at 4°C, under continuous rotation for 16 h, followed by the addition of 1 M ethanolamine pH 8.0 at 22°C for 2 h to block the unoccupied binding sites of the agarose. The gel was washed at least 5 times with the coupling reaction buffers, alternating buffers A and B. Finally it was washed with solution C and small (1-2 ml) chromatography columns were packed. The gels were equilibrated with 20 mM Tris, 0.05% Triton X-100 or Triton N-101 pH 7.4 and then solubilized human thyroid TSH receptor preparation was run through the gels at 4°C. To assess the coupling efficiency, the first cycle wash from the coupling reaction was retained, and absorbance at 280 nm read. The coupling efficiency was of the order of 99%.

ii. Protein Absorption

Solubilized thyroid membrane preparations were allowed to interact with the different immunoabsorbent columns for at least 1 h at 4°C. Usually the sample volume was half of the gel bed volume and was passed through the column two or more times. Non-absorbed material was eluted with 20 mM Tris-0.05% Triton until no protein elution was detected, followed by 20mM Tris- 2M NaCl - 0.05% Triton to detach the specifically immobilized material.

The procedure used was identical to that used in the TSH affinity chromatography method.

After extensive dialysis to remove the NaCl and concentrated under negative pressure, the protein and detergent concentrations were measured and all samples frozen at -70°C until used for the binding assays and electrophoretic analysis.

3. IODINATION OF BOVINE TSH (b-TSH)

Purified bovine TSH (a gift from Dr. Pierce) was iodinated with ^{125}I by the lactoperoxidase method as described by Marchalonis (1969). A 0.5 mCi quantity of Na ^{125}I was incubated with 2.5 µg of b-TSH, 5 µg of lactoperoxidase and 30% H_2O_2, in 0.05 M $NaPO_4$ buffer, pH 7.2, in a total volume of 52 µl, for 5 min at 22°C. The reaction was stopped by the addition of 500 µl of 10 mM Tris, 50 mM NaCl, pH 7.4 containing 1% BSA. The sample was applied to a 10 ml bed volume of Sephadex G-50 gel and eluted with the above solution. The organic peak fractions were pooled and purified on human particulate thyroid membrane preparation as described by O'Donnell et al. (1978). Previously prepared (as described above) thyroid membranes, 10-12 g/Eq, were incubated for 30 min with the ^{125}I-TSH at room temperature and centrifuged at 12,000 x g for 10 min at 4°C. The pellet obtained was washed twice with 20 mM Tris, 50 mM NaCl, 1% BSA, pH 7.4. The last pellet was resuspended in 3 ml of 25 mM Tris, 3 M NaCl, incubated for 30 min at 4°C and centrifuged at 100,000 x g for 1 h at 4°C. The purified ^{125}I-TSH obtained in the supernatant was then applied to a

Sepharose CL 6B column (60-70 ml bed volume) previously equilibrated with 25 mM Tris, 50 mM NaCl, 0.1% BSA, pH 7.4. Aliquots of 0.5 ml were collected and counted. Individual ^{125}I-TSH peak fractions were kept at -20°C, for their use in the binding assay. Usually only one fraction of the peak, corresponding to the highest activity was used.

In some experiments a slight variation of the procedure was used. A 1.5 mCi quantity of Na ^{125}I was incubated with 5 µg of b-TSH, 30 mg of H_2O_2 and 10 µg of lactoperoxidase for 10 min, at room temperature. The reaction was stopped by the addition of 500 µg of 0.1 M PO_4 buffer, pH 7.4 and the reaction mixture applied to a Sephadex G-25 column. After bovine thyroid membrane purification of the labelled TSH, the sample was further purified in a 70 ml bed volume Sephadex G-100, eluted with 20 mM Tris solution, pH 7.4 containing 0.5% BSA. Peak fractions were pooled and aliquoted prior to freezing and storage at -70°C.

a) Calculations to Determine the Specific Activity of ^{125}I-TSH

The radioactivity added in the reaction mixture was counted in a "dose calibrator" for ^{125}I. Fractions of the organic and the inorganic peaks from the Sephadex G-50 column were counted, as were residuals (washed pipette used to transfer reaction mixture to the gel column and the empty reaction tube) and aliquots of the Sephadex G-50 gel.

Calculations were done as follows:

$$\% \text{ of Transfer} = \frac{\text{Organic Peak + Inorganic Peak + gel Counts}}{\text{Total Activity - Residuals}} \times 100$$

$$\mu Ci\ TSH = \frac{\text{Organic Peak} \times \text{mCi Added}}{\text{Total Activity} - \text{Residuals}}$$

$$\mu g\ TSH = \%\ \text{of Transfer} \times \mu g\ \text{of TSH in Reaction}$$

$$\text{Specific Activity} = \frac{\mu Ci\ TSH}{\mu g\ TSH}$$

b) Cholera Toxin Iodination

Purified cholera toxin was iodinated by the chloramine-T method as described by Cuatrecases (1973a). Fifty micrograms of cholera toxin in 10 µl of saline solution were mixed with 125 µl of 0.25 M sodium phosphate buffer, pH 7.4, 20 µl of chloramine-T (2.5 mg/ml) and 10 µl of Na ^{125}I (1.5 mCi). After 30 sec at room temperature, 20 µl of sodium metabisulfite, (5 mg/ml) were added and incubated for 10 sec. The reaction was stopped by the addition of 200 µl of 0.1 M sodium phosphate buffer containing 0.1% of BSA, pH 7.4.

The sample was applied to a Sephadex G-50 column (15 ml bed volume) previously equilibrated with 0.1 M sodium phosphate buffer containing 0.1% BSA, pH 7.4. The radioactivity in the void volume was pooled, aliquoted and then frozen at -70^{o}C.

An aliquot of the iodinated material was precipitated with 10% trichloroacetic acid (TCA) to determine the percent of protein iodinated prior to the application of the sample to the Sephadex column.

c) ^{125}I-bTSH Binding to Particulate Membrane Preparations

Two different binding assays were used:

i. Particulate thyroid membrane preparations (MP) were tested for their capacity to bind ^{125}I - TSH by the assay described by O'Donnell et al. (1978) and Garcia et al. (1982). In microfuge tubes, aliquots of MP usually containing 100 to 250 µg protein in 25mM Tris with 1% BSA, pH 7.2 were incubated with 5,000-20,000 cpm of ^{125}I-TSH in a total volume of 200-300 µl for 30 min at room temperature. Non-specific binding was determined by the addition of excess (10^5 fold) non-labelled TSH (Thytropar). The assay tubes were counted to determine the total counts added. The reaction was stopped by the addition of 0.75 ml of cold 25 mM Tris buffer containing 2.5% BSA. The tubes were centrifuged in a microfuge at 15,000 rpm for 10 min and the pellets counted.

ii. The second type of binding assay for particulate membrane preparations was described by Sato et al. (1977). In this assay, aliquots of MP (protein concentrations 20-50 µg/100 µl) in 20 mM Tris, 0.5% BSA, pH 7.4 were incubated with 50 µl of ^{125}I-TSH containing 10,000 cpm in the presence or absence of excess non-labelled TSH, for 1 h at 37^{o}C in a shaking bath. The tubes were then placed on ice and the reaction stopped by adding 1 ml of cold 20 mM Tris, 50 mM NaCl, 0.1% BSA, pH 7.4. The bound label was separated from free ^{125}I-TSH by centrifugation at 15,000 rpm for 20 min and the pellets counted.

This assay was used for experiments where Scatchard analysis of the data was desired and these samples were incubated with 10,000

cpm of ^{125}I-TSH in the presence of increasing concentrations of cold TSH (0.015-10 mU) in 50 µl of 20 mM Tris, 0.5% BSA. The non-specific binding was determined in the samples containing 100 mU/tube of cold TSH.

In both assays duplicate or triplicate samples were tested.

d) ^{125}I-bTSH Binding Assays to Solubilized Membrane Proteins

Binding of ^{125}I-TSH to solubilized membrane proteins was measured by two different assays as described by Tate et al. (1975) and by Koizumi et al. (1982), respectively.

i. In the first assay, 50-150 µg of solubilized material were incubated with 25 mM Tris, 2.5% BSA, pH 7.2 with or without unlabelled TSH for 15 min at 22°C prior to the addition of 20,000 to 30,000 cpm of ^{125}I-TSH, in a final volume of 0.2 ml. The mixture then was incubated for 45 min at 22°C during which time, tubes were counted to determine the total radioactivity per tube. Normal human IgG (250 µg/tube in 20 µl) was then added followed by a 30% polyethyleneglycol 4000 (PEG) solution to give a final PEG concentration of 15%. Bound and free labelled hormone were separated by membrane filtration. The filters were previously soaked in a Tris solution containing 2.5% BSA for at least 3 h before their use. The filters were placed on a Millipore filter vacuum manifold and kept moist with the 2.5% BSA soaking solution, until used. The reaction mixture from each tube was transferred with pasteur pipetes to individual filters. Each tube was washed three times with a 4% PEG 4000 solution and the washes

were also added to the filters. The filters were then placed into clean tubes and counted. The transfer pipette tips were broken and placed in the appropriate reaction tube. The radioactivity found in these tubes was called the residual count in the calculations. The amount of bound radioactivity was calculated by the following formula:

$$\% \text{ Binding} = \frac{\text{Filter Counts}}{\text{Total Counts} - \text{Residual Counts} - \text{Filter Counts}} \times 100$$

Several variations were done in this assay in order to decrease the non-specific binding and reduce the interassay variability. This will be discussed in the results.

ii. For the second assay, 20 to 50 µg of membrane protein were incubated with 10,000 cpm of ^{125}I-TSH with or without unlabelled TSH (20-30 mU/tube) for 15 min in a shaking bath at 37^{o}C. The total reaction volume was 20 µl and the reagents were in 20 mM Tris containing 0.1 to 0.5% BSA and 0.02-0.03% of Triton N-101, pH 7.4. After this incubation, the tubes were placed on ice and 1 mg of normal IgG in 0.5 ml of 20 mM Tris, 50 mM NaCl, pH 7.4 was added, followed by 0.7 ml of a 30% solution of PEG-4000 in 1 M NaCl, 20 mM Tris, pH 7.4. The tubes were centrifuged at 3,500 rpm for 20 min at 4^{o}C. The supernatants were aspirated and the pellets counted.

For Scatchard analysis, displacement of ^{125}I-TSH was done by the addition of 0.01 mU-10 mU of cold TSH to the incubation mixture.

e) Thyrotropin Binding Inhibition Assay (TBI)

This assay measures the capacity of certain IgGs to inhibit the binding of ^{125}I-TSH to its receptor in different preparations, and is based on the assays described by Mehdi et al. (1973) and by Smith et al. (1974). Assays were performed as for the regular binding assay, but with the addition of different concentrations of IgG from patients with Graves' disease or from a normal population. In assay i for particulate and solubilized membrane preparations, IgG was added before the ^{125}I-TSH and incubated with the TSH receptor preparation for 15 min at 22^{o}C, whereas in assay ii, the IgG was added at the same time as ^{125}I-TSH.

The binding inhibition was calculated as follows:

$$\%TBI = 1 - \frac{\text{(Specific binding of } ^{125}\text{I-TSH in presence of IgG)}}{\text{(Specific binding of } ^{125}\text{I-TSH in absence of IgG)}} \times 100$$

f) ^{125}I-Cholera Toxin Binding Assays

Binding of ^{125}I-CT to TSH-R particulate preparations was done as described for ^{125}I-TSH in binding ii with the only variation being incubation time which was reduced to 5 minutes.

In the solubilized preparations, ^{125}I-CT binding was performed under the same incubation conditions as in binding assay ii of ^{125}I-TSH to solubilized material. Bound ligand was precipitated with equal assay volume of 15% PEG-4000 in presence of 0.5 M NaCl and 500 µl of saline solution (instead of human IgG) at 3,500 x rpm for 20 min.

In all binding assays, the non-specific binding was determined by addition of excess cold CT to control tubes.

4. PURIFICATION OF IMMUNOGLOBULINS (IgG)

IgG from human sera or mice ascites fluid were purified by affinity chromatography using Protein A - Sepharose CL-4B and by chromatography on DEAE cellulose, by the methods of Goding (1976) and James et al. (1964).

Protein A - Sepharose CL-4B, purchased from Pharmacia was used to prepare 5 ml bed volume columns in 0.01 M Na phosphate buffer, pH 7.2 (buffer A). Serum samples were heat inactivated in a water bath at 58^{o}C for 30 min. Purification of IgG was done at room temperature. Aliquots of serum (5 ml or less) were applied to the top of the column and eluted with 150 ml of buffer A or until the eluate gave an absorbance of < 0.02 at 280 nm. Once all the non-bound material was removed, immobilized IgG was eluted with a 0.58% acetic acid solution (solution B) containing NaCl, pH 3. Approximately 90% of the IgG was eluted within the first 10-15 ml. The column was washed with solution B until no absorbance was measured in the eluate prior to re-equilibration of the column with buffer A.

The pH of the eluted IgG was adjusted to 7.2 with 2 M Tris solution, prior to concentrating the sample under negative pressure in Minicon concentrators. Samples were dialyzed for 24 h at 4^{o}C against 25 mM Tris, pH 7.2. Protein concentration was estimated at 280 nm and samples were kept at -20^{o}C until used.

Protein determination was calculated by the following formula:

$$\text{Protein (mg/ml)} = \text{Absorbance reading 280 nm} \times \frac{41}{1.41}$$

considering 1.41 the absorbance at 280 nm of 1 mg/ml of IgG protein.

a) DEAE Cellulose

IgG purified by this method was usually previously partially purified by 40% ammonium sulphate precipitation (1.64 M) as described by Kendall (1937). Serum or mouse ascites containing monoclonal antibodies were incubated with $(NH_4)_2SO_4$ for 20 min at 22°C followed by centrifugation at 2,000 x g. The pellet was dissolved in distilled deionized water and dialyzed for 1 h against d-d H_2O and for 24 h against 20 mM Tris or saline solution. The sample volume was kept as 1/2 of the original serum sample volume.

Partially purified IgG samples were then applied to a DEAE column previously equilibrated with 50 mM Tris, pH 8.0 (10 ml sample/30 ml bed volume DEAE). IgG elutes in the first protein peak after the void volume. DEAE cellulose was then washed with 0.5 N NaOH and 0.5 N HCl to remove other plasma proteins immobilized on it (Sober, 1956).

b) Fractionation of IgG

Fab fragments were dissociated from Fc by papain hydrolysis as described by Porter (1959).

DEAE purified IgG was incubated with 1 mg papain/100 mg IgG in 0.4 M phosphate buffer containing 40 mM cysteine and 8 mM EDTA, pH 7 for 12-18 h at 37°C. The sample was then dialyzed against 10 mM phosphate buffer, pH 7.6 for 24 h and fractionated on a carboxymethyl (CM) cellulose column eluted with 10 mM phosphate buffer, pH 7.6 (Fc fragment) and then with 10 mM phosphate, 0.4 M NaCl, pH 7.6 to obtain the Fab peak.

Both peaks were concentrated and dialyzed against 0.1 M phosphate buffer, pH 7.0 and applied to Sepharose Protein A columns. Purified Fc fragment was eluted from the Protein A gel with 0.1 M glycine, pH 2.8 whereas the Fab fragment was eluted with phosphate buffer. Alternately, peaks obtained from the CM cellulose column were also further purified on a DEAE column.

$F(ab')_2$ fragments were obtained by pepsin digestion of the IgG preparation as described by Stanworth (1978). IgG was dialyzed against Walpole's acetate buffer (0.1 M Na acetate, pH 4.1 with glacial acetic acid). Pepsin was dissolved in the same buffer and incubated (1 mg/100 mg IgG) with IgG at 37°C for 24 h. The incubation was stopped by the addition of solid Tris until the sample pH was 8.0. The neutralized sample was applied to a Sephadex G-150 column equilibrated with 100 mM Tris, 200 mM NaCl, 2 mM Na EDTA, pH 7.6. $F(ab')_2$ fragments were collected in the mid part of the first protein peak and were further separated from intact IgG by fractionation on a Sephadex G-100 column.

5. MOUSE IMMUNIZATION

Balb C mice 12 weeks or older, were immunized with b-TSH affinity chromatography purified TSH receptor preparation, solubilized (SDOC) thyroid membrane proteins, particulate purified human thyroid membranes or guinea pig fat cell membranes. Injections were given at weekly or longer intervals, intraperitoneally or in the foot pads. Protein concentrations of the samples injected were variable, but were kept constant for a given type of immunization. The first injection was given in complete Freund's adjuvant whereas the subsequent injections used incomplete Freund's adjuvant.

Each animal received at least 3 injections prior to sacrifice for the fusion, and all were tested for antibody response to the given antigen prior to the last injection. Fusions were done 3 to 4 days after the last booster injection. To test the mouse antibody production blood was obtained by making a small cut in the tail. Immunized mice sera were tested in parallel with non-immunized mice sera of the same strain and age, in the screening assay.

a) Fusions and Production of Monoclonal Antibodies

Spleens from immunized animals were fused with mouse myeloma cells following the technique of Kohler and Milstein (1975). Myeloma cells used in the fusions were the MOPC 315.43 line, which contains both a resistant and a sensitive [hypoxanthine phosphoribosyltransferase (HPRT), ouabain] marker as described by Mosmann et al. (1979).

The mice were sacrificed and the spleen aseptically excised and the capsule removed. Spleen cells were kept in RPMI 1640 medium containing 50 µM 2-mercaptoethanol (2ME), 5% foetal calf serum (FCS) and 20 µg/ml, lipopolysaccharide (LPS), at 37°C in 7.5% CO_2 incubator until a fusion was set up usually within 24 h. When a fusion was carried out immediately following splenic removal, LPS was not added to the medium.

Spleen cells and myeloma cells were washed and mixed in a ratio 5:1. After centrifugation at 900 x g for 10 min the pelleted cells were fused by the addition of 2 ml of PEG 1540 (40% w/v), followed by the addition of medium containing 10% FCS to partially resuspend the pellet. Cell clumps were incubated for 1-3 h at 37°C and finally resuspended in medium containing the following: 7% FCS, 10^7 mouse red blood cells/ml, 50 µM 2ME, 100 µM hypoxanthine, 30 µM thymidine, 0.5 µM methotrexate, 1 mM ouabain, and 50 µg/ml gentamycin. The suspension was aliquoted into 96-well Linbro trays and allowed to grow at 37°C in 7.5% CO_2 until clones could be visualized, usually within 10-14 days (modification of the method of Galfre et al. 1977).

The cells were tested for the presence of antibodies in the media as described in "screening assays for monoclonal antibodies".

b) Screening Assays for Monoclonal Antibodies

Hybrid cells were tested for the production of antibodies against human thyroid and guinea pig fat cell membrane antigens. Media from growing clones were generally first tested for presence of IgG before the antigen specific test was done.

The detection of IgG was assessed by a modification of the method of Slaughter et al. (1980). Diluted (1:1000) rabbit antimouse IgG from Coggel company was aliquoted in flexible polyvinylchloride microtitre plates (PVC plates) and incubated for 1 h at 37°C or for 4 h at 22°C or overnight at 4°C. Wells were emptied of remaining fluid and washed three times with PBS containing 1% BSA, 0.1% gelatin and 0.02% NaN_3. To reduce the background, binding wells were filled with the same buffer and incubated for 1 h at 22°C, after which they were emptied and 50 µl of diluted media (1:10 with the above buffer) was added and incubated for 1 h at 22°C. After washing the plate, 50,000-100,000 cpm of ^{125}I sheep antimouse IgG were added and incubated for 45 min at 22°C. Wells were washed again with the above buffer, dried, cut and counted after placing each well in a test tube.

Controls were made for each assay by substituting the clones' media by media in wells that had no clones growing on microscopic examination. Background counts were obtained from the average counts detected in control wells multiplied by 1.5. Any media giving higher counts than background was tested for the presence of antigen-specific antibodies.

c) Antigen Specific Screening Assays

Assay i: Solubilized membrane proteins as antigen. In this assay the PVC wells were coated with 10 µg of solubilized thyroid membrane proteins in 40 µl of 20 mM Tris 0.05% Triton N-101 and 0.5% BSA, pH 7.4 and incubated uncovered for 6 h at 37°C

in a drying oven. When the solubilized fat cell membrane proteins were used as an antigen, the protein concentration was 20 µg in the same volume solution. Wells had to appear dry before the addition of the test media or mouse serum. The test material (25 µl) was incubated for 1 h at 37°C in a humidified incubator. Plates were then washed with 20 mM Tris, 50 mM NaCl, 0.1% BSA, pH 7.4 prior to incubation with ^{125}I-sheep antimouse IgG for 45 min at 22°C. When possible, samples were tested in duplicate or triplicate. Controls containing media from negative wells were included. When mouse serum was tested, a non-immunized mouse serum was used as a control.

Assay ii: Particulate guinea pig fat cell membrane. This assay measured the thyrotropin binding inhibition capacity (TBI) of the tested medium. Maximum binding was obtained by the addition of control medium and ^{125}I-TSH. Non-specific binding was included in every assay by adding 50 mU of cold TSH/well. In this assay, non-solubilized material was incubated for 5 h at 37°C in PVC plates (20 µg/25 µl in 20 mM Tris, 0.5% BSA, pH 7.4), followed by aliquots of mouse sera or clones' media previously mixed with 10,000 cpm of ^{125}I-TSH (specific activity 80-100 µCi/µg). The incubation was at 37°C for 1 h. Wells were then washed 3 times with 20 mM Tris, 50 mM NaCl, 0.1% BSA, pH 7.4.

Other assays for the detection of IgG that could inhibit the binding of ^{125}I-TSH to receptor preparations were also tested. Among them, poly-L-lysine coated PVC microtiter plates and direct binding of IgG to the plates were exhaustively tested. These assays will be discussed in the results.

d) Expansion of Positive Clones and Production of Large Amounts of Monoclonal Antibodies

Once a positive clone was detected, cells were subjected to limiting dilution so as to isolate a single cell. Occasionally when only one clone per well was observed limiting dilutions were not performed. Sub-cloning of the positive clones was tested again and positive cells were allowed to grow for several days until reaching a few million cells. IgG was obtained from the clones' medium or by ascites production in irradiated and 2, 6, 10, 14-tetramethylpentadecane (Pristane) primed mice.

e) Mouse Production of Monoclonal IgG in Ascites

Balb C/Cr mice were primed with 0.5 ml of Pristane, given subcutaneously 4-14 days prior to irradiation with 500 rads. One to six million hybrid cells were injected intraperitoneally per mouse. Cells were suspended in PBS or media. Usually ascites could be collected 5 to 7 days post injection.

The ascitic IgG was partially purified by 1.64 M ammonium sulphate precipitation as described in "Purification of Immunoglobulin G".

6. ASSAYS TO DETERMINE THE PRODUCTION OF CYCLIC ADENOSINE MONOPHOSPHATE

Cyclic adenosine monophosphate (c-AMP) production in thyroid slices was determined by the method of Zakarija et al. (1980c).

Human thyroid tissue was obtained at surgery, cut in 1 to 2 mm thick slices weighing between 4 and 15 mg, with a Stadie-Riggs microtome and kept ice cold in a Krebs-Ringer-bicarbonate-glucose albumin buffer.

The IgGs' to be tested were $(NH_4)_2SO_4$ (1.64 M) and/ or DEAE purified in a 0.9% NaCl solution, and 300 µl of each were placed into small capped glass vials. Krebs-Ringer-bicarbonate buffer (300 µl), pH 7.4 containing 0.2% glucose, 0.2% human serum albumin and 20 mM theophylline were added per vial. Samples were tested in triplicate or quadruplicate. The assay's concentration of IgG was 1.5 fold that of the original serum concentration.

Vials containing IgG or TSH control were placed in a 37°C water bath under continuous shaking. Each vial was gassed with 95% O_2 5% CO_2 for 30 sec, during which time a slice of thyroid tissue was weighed and then placed in the individual vial. The incubation was carried out for 2 h at 37°C after which, tissue from each vial was removed and immediately homogenized in individual glass homogenizers, one every 30 sec. Homogenization was done in 0.5 ml of cold 6% trichloroacetic

acid. Homogenizers were centrifuged at 3,500 rpm for 20 min at 4°C. Supernatents were transferred to glass tubes and washed three times with 5 ml volume of water saturated ether and evaporated to dryness.

For cAMP determinations a Schwartz-Mann RIA kit was used. The samples were dissolved in 0.05 M sodium acetate buffer pH 6.2.

7. THYROTROPIN RECEPTOR IDENTIFICATION

a) "Western Blots" of Thyroid Membrane Proteins

Blotting of solubilized thyroid membrane proteins and detection with antiserum and ^{125}I-Protein A or ^{125}I-sheep anti-mouse IgG (^{125}I-SAM) was done by the method of Renart et al. (1979) and Towbin et al. (1979).

Proteins were separated by polyacrylamide slab gel electrophoresis (under dissociating or non-dissociating conditions) and transferred to nitrocellulose paper (NC paper) in a blotting chamber (Bio-Rad) at 250 mAmp for 5 hours.

Gels were divided in two portions of which one was stained with Coomassie blue for control of protein mobility. The gel to be transferred was placed in contact with wet NC paper and regular filter paper on the other surface, and the sandwich placed between two sheets of scotch bright soaked in blotting buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) and a plastic perforated sheet to hold the sandwich and maintain the NC paper in tight contact with the gel.

After the transfer was finished, gels were stained with silver-stain. NC paper was incubated with the antibody to be tested (human

IgG or monoclonal antibodies) diluted in buffer 1 [150 mM NaCl, 50 mM Tris, 5 mM EDTA, 0.25% gelatin (w/v) and 0.05% Nonidet P.40, pH 7.4] for 5-12 h at 37°C, under gentle rocking. The NC paper was then washed twice with the same buffer without IgG for 2 h at 37°C and 1.2-1.5 µCi of ^{125}I-Sheep anti-mouse (^{125}I-SAM) or ^{125}I-Protein A in buffer 1 were incubated with the paper for 2-3 h under the same conditions.

To detach the non-specifically bound labelled material, the paper was washed with buffer 2 [1 M NaCl, 50 mM Tris, 5 mM EDTA, 0.25% gelatin (w/v) and 0.4% Sarkosyl (w/v) pH 7.4] for 2 to 12 h also at 37°C.

NC paper was dried at room temperature prior to autoradiography with X-Omat Kodak film.

b) Autoradiography

Dried slab gels or blotted nitrocellulose paper were placed in contact with X-Omat film (Kodak) and DuPont Cronex Lightning Plus XL intensifying screen for 24 h to 7 days in a Kodak autoradiography holder tightly covered with aluminum foil. Exposure was at -70°C or at room temperature followed by film developing with D-19 Kodak developer for 8 min and fixation (Kodak rapid-fix) for 4 min in the dark. (Laskey 1977).

c) Rat Thyroid Epithelial Cell Culture

A rat thyroid cell line culture (FRTL) (Ambesi-Impionbato, 1980) was used to study the TSH-receptor by both binding and stimulating activity of TSH and IgG (cAMP production and ^{125}I-TSH inhibition of binding).

The FRTL cell line was found to secrete physiological amounts of thyroglobulin into the culture medium and to concentrate iodine 100-fold. The culture medium is a modification of the F-12 medium, containing insulin, TSH, transferrin, hydrocortisone, somatostatin and glycyl-L-histidyl-L-lysine acetate, (F-12 + 6H).

The cells grow in clumps which remain attached to the culture dish. When the required cell density was achieved, culture media was changed to F-12 + 5H (as above culture media without the TSH) and 3 to 7 days later the experiments were performed.

i. IgG Activity

IgG from patients with Graves' disease and monoclonal antibodies raised against human thyroid membrane preparations were tested in the FRTL cell line to assess stimulation of cAMP production and to measure inhibition of binding of ^{125}I-TSH to these cells in culture.

ii. cAMP Production

FRTL cells were allowed to grow in 24 well Linbro plates. When the cell density was approximately 75% confluent the F-12 + 6H medium was changed to medium F-12 + 5H. The cells were extensively washed with PBS or Hanks solutions, and incubated for 2 h at 37^{o}C in 5% CO_2, 95% O_2 with different dilutions of the IgGs

tested. Stimulation of cAMP by TSH (10 mU/ml) was always included as a control in every Linbro plate.

After aspiration of the medium, 0.3 ml of cold ethanol was added to each well and the plates were left at -20°C for 16 h wrapped in Saran wrap. The ethanol was then transferred to glass tubes and dried under N_2 for 20 minutes. The tubes were kept in a desiccator until the measurement of cAMP content by RIA was done.

iii. ^{125}I-TSH Binding Inhibition

FRTL cells in F-12 + 5H were incubated with different concentrations of human IgG or cholera toxin or dilutions of monoclonal antibodies in ascites fluid or culture medium. Except for the monoclonal antibodies, the IgGs and cholera toxin were in saline solution containing 0.1% BSA. The test samples (125 µl) were mixed with ^{125}I-TSH in 20 mM Tris, 0.5% BSA (≈25,000 cpm in 375 µl), pH 7.4 prior to incubation with the FRTL cells.

Cells were washed with the same solution (Tris-BSA) and 200 µl of the above mixture was added per well. The incubation period was at 37°C in 95% or 5% CO_2 for 1 h. After 2 washes with the above solution containing 50 mM NaCl, the cell membrane proteins were solubilized with 300 µl of 1% Nonidet P.40. The solubilization was carried out under the same conditions for 2 h and the test sample was then transferred to test tubes and radioactivity counted.

Every experiment (each plate) included a maximum binding (no IgG added) and a nonspecific binding (50 mU TSH/well). All samples were tested in duplicate.

d) TSH-Receptor Studies in FRTL Cells

FRTL cells in F-12 + 5h were labelled with ^{32}P and then, the membrane proteins were solubilized, precipitated with normal or Graves' IgG, and analyzed by electrophoresis and autoradiography.

i. Phosphorylation Procedure

Cell phosphorylation was done by the method of Kasuga et al. (1982) with some modifications.

FRTL cells were washed extensively with phosphate-free medium (PO_4-free MEM medium) and incubated with the same medium for 30 min before starting the experiments. The ^{32}P was diluted in the above medium and gassed to achieve an approximate pH of 7.4. One to two million cells were incubated with 100-200 µCi of ^{32}P for 1 h at 37°C in 5% CO_2 in presence or absence of TSH (50-500 mU/well). In some experiments TSH was added to the incubation mixture 1 h after ^{32}P, and further incubated for 5-15 min.

The phosphorylation reaction was stopped by dilution and 3 washes with F-12 media containing 50 mM Na pyrophosphate, 10 mM NaF and 10 mM EDTA. Immediately after, cells were lysed with a solution of 20 mM Tris, 1% Triton N.101 and 2 mM PMSF. Solubilization was carried out at 24°C for 1 h. The well fluid was collected and centrifuged at 100,000 x g for 1 h to precipitate any non-solubilized material.

ii. Thyroid Membrane Protein Precipitation

^{32}P-labelled solubilized material was precipitated with normal or Graves' IgG by the methods described by Kessler (1975) and

Kozlovskis et al. (1982). Alternatively, in some experiments goat antihuman IgG was used instead of Protein A.

The ^{32}P labelled solubilized material (1.5 ml) was incubated with 50 µl of a 10% suspension of Protein A at 24°C for 30 min, followed by centrifugation at 5000 x g for 10 min. Aliquots of the supernatant were incubated with normal or Graves' IgG for 1 h at 4°C with frequent mixing. Protein A was added to achieve a final concentration of 1% and the mixture was incubated for 30 min at 24°C, centrifuged at 5000 x g for 10 min and the pellets washed twice with 150 mM NaCl, 5mM EDTA, 50 mM Tris, 0.02% NaN_3 and 0.05% Triton N-101, pH 7.4.

The final pellet was resuspended in 100 µl of 1% SDS, 9.5 M Urea and 10% 2ME, boiled for 2 min and centrifuged again at 5000 x g for 10 min. The resulting supernatants were centrifuged one last time in an aerofuge at 90,000 x g for 10 min prior to the precipitate being subjected to electrophoresis. The precipitated proteins were analyzed in SDS-PAGE as described above and the dried gels autoradiographed. Autoradiograms were scanned in a gel scanner.

In one experiment, precipitated proteins were submitted to two dimensional gel electrophoresis (isoelectricfocusing followed by SDS electrophoresis) as described by Kozlovskis et al. (1982); for this assay, samples were solubilized in the above described solution and mixed with 35 µl of a solution containing 4 ml of 10% Nonidet P.40 (NP.40), 80 µl of 3/10 ampholines, 160µl of 5/7 ampholines, followed by the addition of 140 µl of 9.5 M Urea containing 2% NP-40, 5% 2ME, 0.4% 3/10 ampholines, 0.8% 4/10

ampholines and 0.8% 5/7 ampholines. Samples of 150 to 200 µl were loaded directly onto cylindrical gels of 3% polycrylamide containing 6% ampholines. The second dimension was on 10% SDS-PAGE as described above. Gels were stained with silverstain and autoradiographed.

CHAPTER III

PURIFICATION OF THE THYROTROPIN RECEPTOR (TSH-R)

1. INTRODUCTION

The IgG responsible for the abnormal stimulation of the thyroid gland in patients with Graves' disease is supposed to bind to the cell membranes through the TSH-R. This hypothesis is mainly based on the fact that binding of ^{125}I-TSH to solubilized thyroid membrane protein preparation is inhibited in the presence of IgG from these patients (Petersen et al. 1977, Dawes et al. 1978, Rees Smith et al. 1980, Rickards et al. 1981, Kotulla et al. 1981). However, no other proof exists at present to confirm this hypothesis for which the isolation of TSH-R would be necessary.

Although the thyroid stimulating antibody (TSAb) has not been completely purified, studies have indicated that it also binds to other subcellular fractions of the thyroid homogenates (Dawes et al. 1978) to which no ^{125}I-TSH binding can be detected. Fenzi et al. (1979b) also found that IgG from patients with Graves' and Hashimoto's diseases could bind to thyroid membrane preparations devoid of TSH-R. However, since patients with Graves' disease have abnormal amounts of thyroglobulin and microsomal antibodies (Mori et al. 1971) and since the TSAb are not pure, contamination of the IgG preparations could account for the results observed.

The presence of the specific antigens for Tg and microsomal antibodies in cell-mediated immunity studies will mask the results, since not only the effects caused by the specific antigen of TSAb would be produced, but also the interaction of the other antigens and peripheral lymphocytes could be observed. In view of the possibility of an immunologic derangement in these patients (Volpe 1978), such studies are of major interest to further elucidate the pathogenesis of Graves' disease.

Finally, even if the TSH-R is, indeed, the true antigenic site for Graves' IgG, studies would have to be conducted to investigate the possibility of minimal TSH-R alterations in Graves' disease that could trigger the immunological response observed in such patients.

These findings and prospects reinforce the need to obtain a pure preparation of TSH-R with which to investigate the possible antigenic role in the etiology of Graves' disease.

To this end, TSH-R preparations were made from human thyroid tissue and solubilized as described in the methods. Every preparation was standardized for its ^{125}I-TSH binding capacity. Affinity chromatography described by Cuatrecasas et al. (1971) has been successfully used in the purification of proteins and receptors. Specific ligands, such as steroid or polypeptide hormones covalently bound to agarose beds have provided stable gels with which different receptors have been greatly purified (Grandics et al. 1981, Karlin et al. 1976, Dufau et al. 1975, Shorr et al. 1981). Less specific affinity chromotography ligands, such as lectins (Nexo et al. 1979, Roche et al. 1975) or polyclonal antibodies (Kessler 1976, Harrison et al. 1980) have resulted in variable degrees of glycoprotein purification dependent on

the samples' initial purity. Since the starting thyroid material was a crude membrane preparation, TSH was used as a ligand.

Earlier attempts to purify the TSH-R from human and other animal sources (bovine and porcine) have given variable results. The authors used TSH as a ligand (Tate et al. 1975, Dawes et al. 1978, Fenzi et al. 1978, Koizumi et al. 1982), or immobilized lectins (Kuzuya et al. 1980). Various detergents were used to solubilize the thyroid membrane proteins which, due to their different contaminants (Chang et al. 1980 and Ozawa et al. 1979a) and critical miceller concentration (Furth 1980), could have contributed to the variations reported with regards to the TSH-R characterization. In most reports however, there is no mention of the contaminants present in the purified TSH-R preparations and degree of purification is only indicated by ^{125}I-TSH binding measurements.

Because of the above mentioned reasons and the impossibility at the present state of knowledge to identify the TSH-R, studies were conducted to detect the presence of other thyroid protein molecules in the partially purified preparations and to partially characterize the TSH-R.

Although significant purification of the TSH-R was obtained with the TSH-affinity chromatography, contaminants such as thyroglobulin, were still present. In an attempt to separate this large antigenic molecule from the TSH-R, immunoabsorbent gels were made with DEAE-purified IgG from patients with Hashimoto's thyroiditis and Graves' disease. Solubilized thyroid membrane preparations were applied to the first column (the patient had a very high thyroglobulin antibody titre) and the eluate adsorbed by the second immunoabsorbent. With

this approach, not only thyroglobulin adsorption was attempted, but also this allowed one to determine whether the TSH-R was specifically absorbed by the Graves' IgG.

2. RESULTS

a) TSH-R Preparations, TSH Iodination and Binding Assays

i) Human Thyroid Membrane Preparations

Particulate TSH-R preparations obtained as described from post--mortem or surgical human thyroid tissues were studied by electron microscopy. The purified membrane preparation (obtained by sucrose gradient sedimentation) showed predominance of cell membrane structures whereas the crude membrane preparation contained more intracellular organelles (not shown).

Comparison of the two particulate preparations (Garcia et al. 1982) demonstrated that, when both were made from the same tissue so as to obtain the same ^{125}I-TSH specific binding, the protein concentration of the crude membrane preparation had to be 3 times higher than the protein concentration in the purified preparation. However, recovery of total protein was 10 times less in the purified preparation than in the crude, implying a considerable loss of TSH-R during the membrane purification. This receptor was recovered in the pellet of the first sucrose gradient centrifugation, which was discarded in the usual procedure. Since the purified membrane preparation did not improve the results obtained with the crude membrane preparation and

the procedure was 3 times longer, only the former was used for the TSH-R purification, isolation and for most of the binding assays to study the interactions of TSH-R with TSH and IgG.

Protein concentrations (Bradford method) of the crude particulate thyroid preparations were variable (250-2000 µg/100 µl) giving a total protein recovery between 2-5 mg per gram of thyroid tissue. Estimation of protein concentration was done by linear regression obtained from the standard curve using bovine-serum albumin (BSA) as standard protein (BSA E_{co}^{280} 0.7 = 1 mg/ml). The correlation coefficient for the standard curves was 0.980-0.999.

The presence of TSH-R in the particulate preparations was assessed by binding to purified ^{125}I-bTSH. Bovine TSH was iodinated to a specific activity between 50-90 µCi/µg. Sepharose CL 6B chromatographic profile (Fig. 1) and Sephadex G-100 profile were identical.

Because differences in specific binding to the TSH-R preparations were observed with the various fractions obtained in the major radioactive peak, only a few of them were used in the binding studies. The first fractions of the peak gave higher non-specific binding (binding non-displaceable with excess cold bTSH), whereas fractions containing the maximal radioactivity and those on the descending part of the curve gave lower total binding, but higher specific binding to TSH-R preparations.

Using the described binding assays, the TSH-R preparations had saturable binding activity specific for ^{125}I-TSH (Fig. 2 & 3). Other polypeptide hormones such as prolactin, LH, FSH and insulin, also labelled with ^{125}I by the same method, did not show any specific

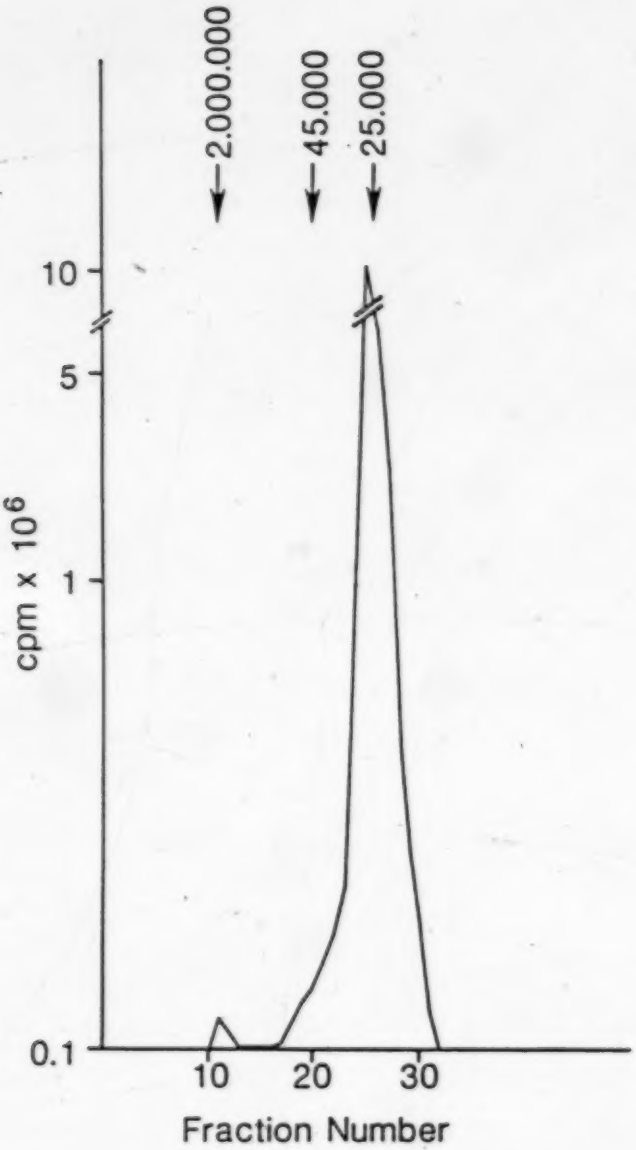


Figure 1. Sepharose CL6B chromatographic elution profile of ^{125}I-bTSH purified on human thyroid membranes. Arrows indicate elution of standard proteins. The ^{125}I-bTSH was purified as described in Methods.

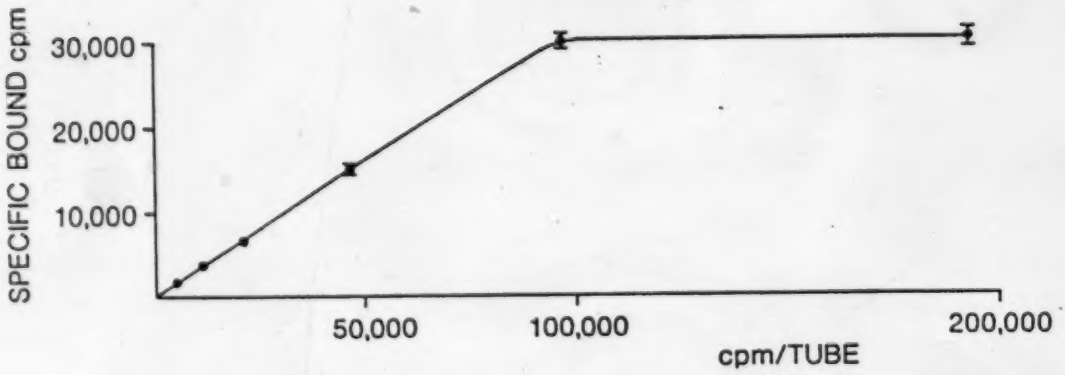


Figure 2. ^{125}I-TSH binding to particulate thyroid membrane preparations. Binding of increasing concentrations of ^{125}I-TSH 250 µg of human crude particulate thyroid membrane preparation is shown. Binding assay was carried out at 24°C for 30 min under constant shaking.

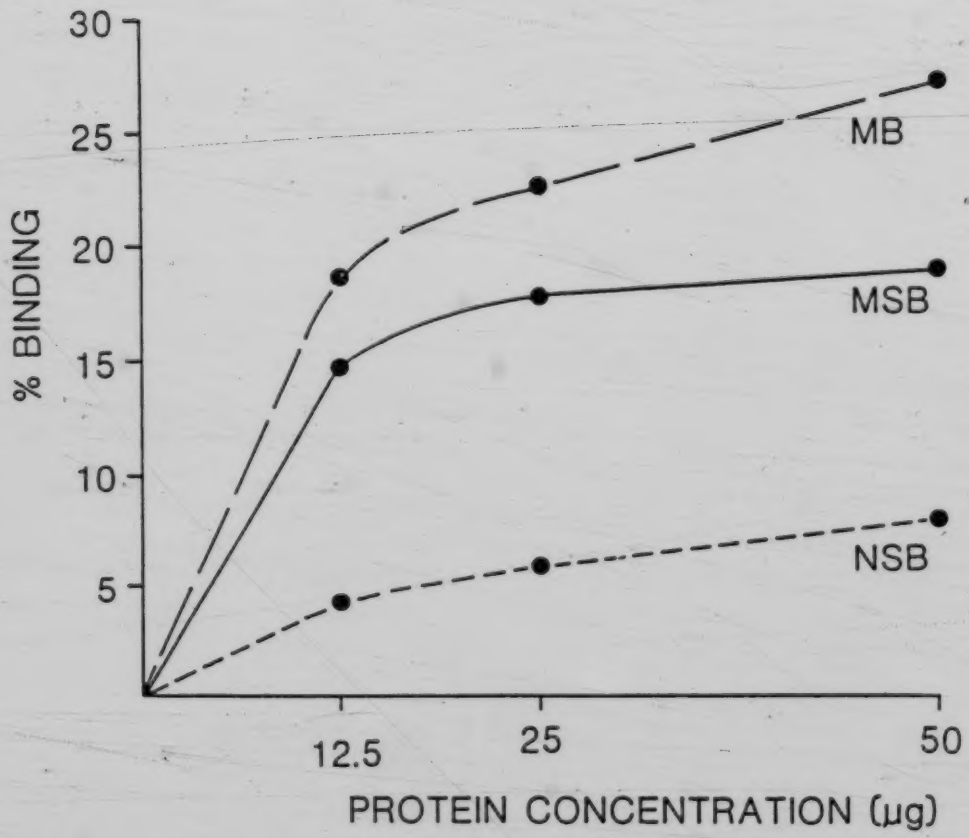


Figure 3. ^{125}I-TSH binding to increasing concentrations of human thyroid membrane (µg/200 µl). Increasing concentrations of human crude particulate thyroid membrane protein binding to 10,000 cpm of purified ^{125}I-TSH are shown. Binding assay was carried out at 37^{o}C for 60 m, under constant shaking. MB = maximum binding, MSB = maximum specific binding, NSB = non-specific binding.

binding to the same membrane preparation (Fig. 4); nor did thyroglobulin, T_4 or T_3.

Scatchard analysis of the crude thyroid membrane preparation (Fig. 5) showed the presence of a high affinity binding site with a Ka of $1.18 \times 10^9 M^{-1}$ and a binding capacity of 102 pg bTSH per microgram of protein. A low affinity binding site was also present.

Although a comparative study of the two binding assays for crude membrane preparations was not done, it was observed that binding at 24°C for 30 minutes required higher (2-5 times) concentrations of thyroid membrane protein than binding at 37°C for 1h to achieve similar specific binding. Since less ^{125}I-TSH was required for the latter, this assay was subsequently used.

ii) TSH-R Solubilization

As mentioned previously, 3 detergents were employed for solubilization of the TSH-R. Sodium-deoxy-cholate (SDOC) was utilized primarily to solubilize those preparations used in immunization procedures to produce monoclonal antibodies. Several problems were encountered with this detergent, the most important being a considerable loss of ^{125}I-TSH binding to such preparations and the formation of a precipitate in the presence of high concentration of NaCl (3 M) needed for the elution of the TSH-R from the TSH affinity chromatography columns. However, since specific binding was still found with the affinity chromatography purified material, this procedure was used and a few mice were immunized with these preparations.

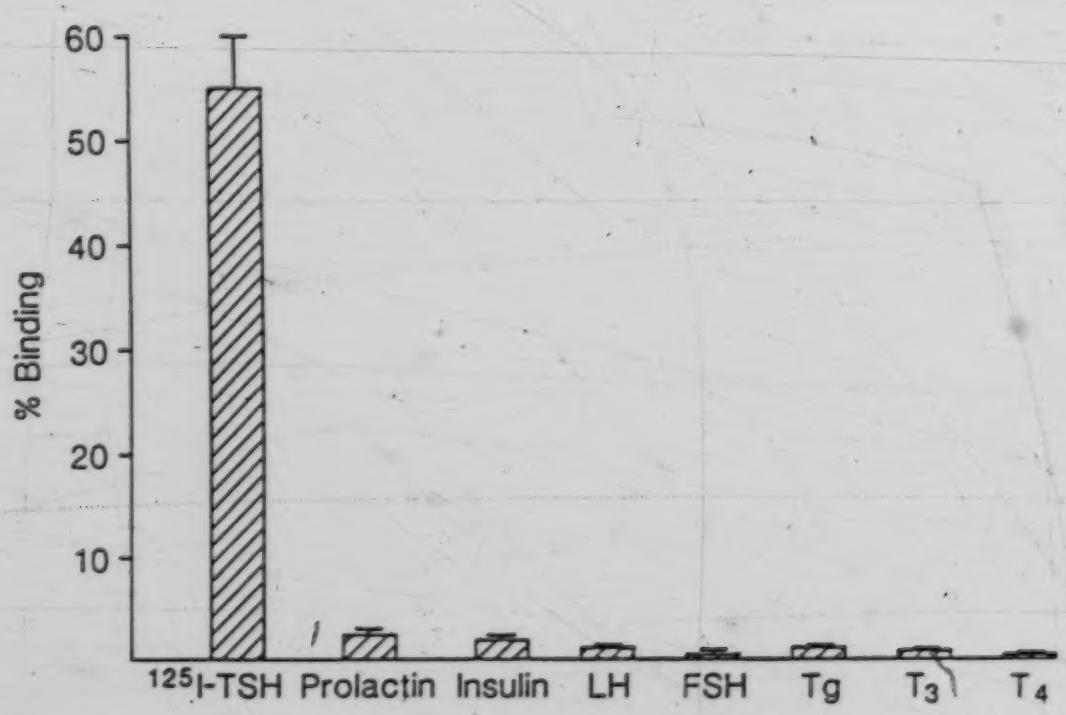


Figure 4. Comparison of the binding of polypeptide hormones to human crude particulate thyroid membrane preparation. Each hormone was iodinated with ^{125}I as used in the radioimmune assays (RIA).

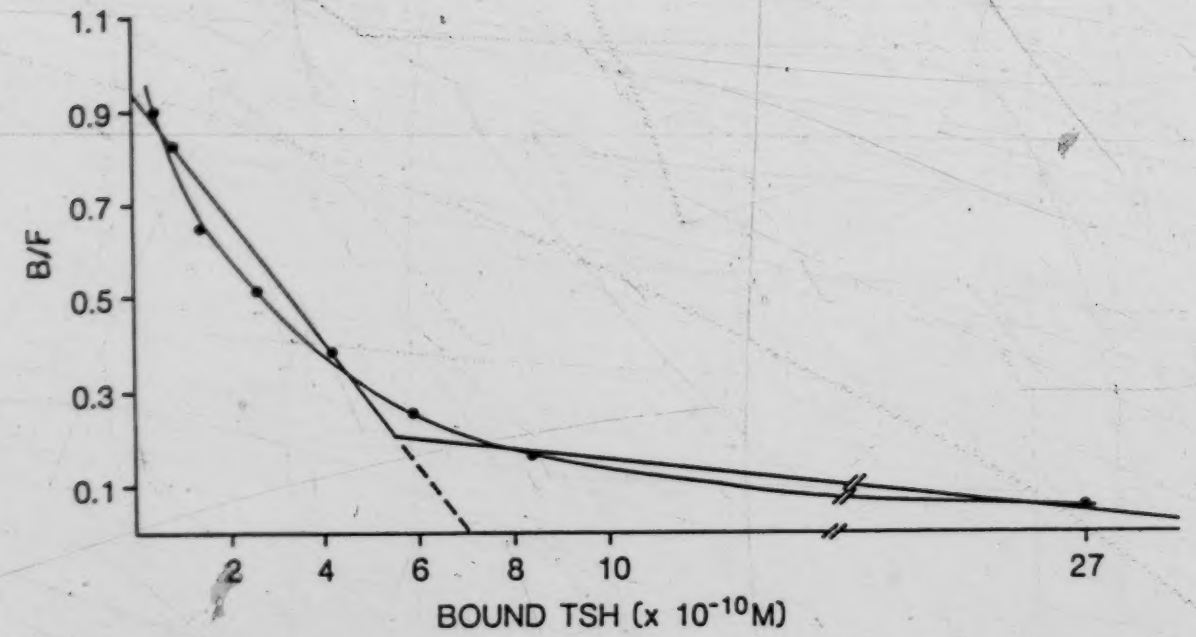


Figure 5. Scatchard plot of specific ^{125}I-TSH binding to human particulate thyroid membranes. Bindings were done at 37°C for 60 min, using increasing amounts of non-labelled TSH (0.007-50 mU/100 µl) and constant ^{125}I-TSH (10,000 cpm = 4 x 10^{-11} IU) in a total assay volume of 200 µl.

For the binding studies and purification of the TSH-R, Triton X-100 or Triton N-101 was used to solubilize the TSH-R from human thyroid and guinea pig fat cell membrane preparations. Results were very similar, if not identical, between the two detergents and the use of either one depended on their availability. The yields obtained per mg of protein varied between 40 and 60%, depending on the starting membrane preparation used. Detergent concentrations were measured prior to determining the protein content of a sample, since the presence of detergent interferred markedly with the protein estimations (Fig. 6). The samples used for the standard curve of the protein determination assay always contained the same concentration of detergent as the unknown with dilutions being made with the same detergent concentration. Since high detergent concentrations interfere with both the binding of ^{125}I-TSH to the TSH-R preparations, and its characterization, Bio-beads SM-12 (Bio-Rad) or Amberlite XAD-2 resin columns were used to remove the excess detergent. To monitor the amount of detergent removed by the resins, ^{3}H-Triton X-100 was used in the initial studies. It was necessary for a sample of 3 ml in 1% Triton X-100 to be in contact with the beads for a total of 1h, in order to extract 99.5% of the Triton X-100 in a 4 ml Bio-bead SM-12 column. The procedure using this resin was done at 24^{o}C. The remaining concentration of detergent (0.05%) was found to be optimal for the binding assay. With Amberlite XAD-2, in a 40 ml bed volume column, 90-95% of the detergent could be removed from a 12-15 ml sample containing 1% Triton N-101 in 45 min at 4^{o}C.

Binding of ^{125}I-TSH to the solubilized TSH-R preparations using the membrane filtration system for separation of free and bound hor-

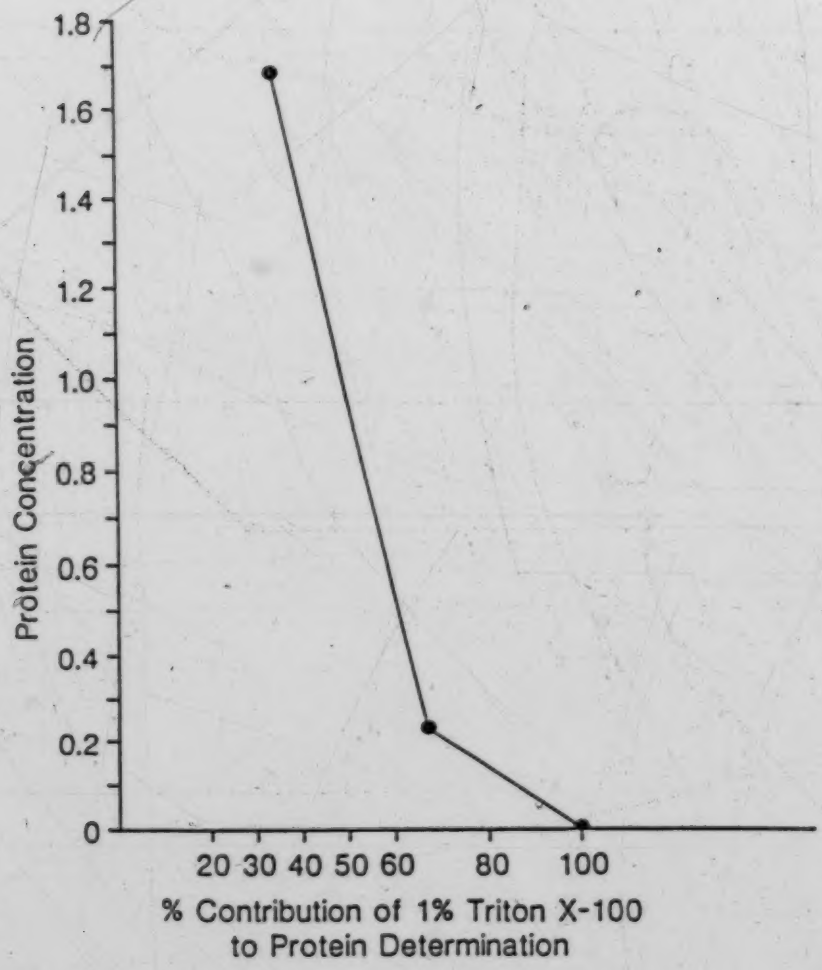


Figure 6. Effects of 1% Triton X-100 on determination of protein concentrations. Solutions of known protein concentration (mg/ml) were mixed with Triton X-100 to a final concentration of 1% (v/v) and protein concentrations determined again.

mone, gave very variable results apparently due to the membrane filters. In some circumstances, up to 25% of the ^{125}I-TSH would bind to the filters in the absence of receptor and could not be detached by repeated washes.

Some variations of the procedure were investigated in an attempt to improve the reproducibility of the asssay and to reduce the non-specific binding. Initially, variable concentrations of human IgG were tried without success to co-precipitate the TSH-R-TSH complex. Different concentrations of PEG in the presence or absence of 0.2 - 1 M NaCl also failed to improve the results. All these studies were done at 24^{o}C with incubations of 45 min.

The solubilized membrane binding assay of Koizumi et al. (1982) using 30% PEG 4000 precipitation of TSH-R-^{125}I-TSH complex, gave lower non-specific binding (5-10%), good reproducibility and an intra-assay variation of 4.0%.

The binding of ^{125}I-TSH to solubilized TSH-R preparations was specific and saturable. As can be observed in Fig. 7, solubilized Graves' thyroid preparation had the highest capacity for specific binding compared to preparations from normal human thyroid and guinea pig fat cell TSH-R. Scatchard analysis of the binding data (Fig. 8 and 9) verified, as in the particulate preparations, the presence of 2 binding sites with differing affinities. The high affinity binding site in the thyroid TSH-R preparation had a Ka of 3.07×10^{9} M^{-1} with a capacity of 9.33×10^{-9} moles/mg protein. In the solubilized guinea pig fat TSH-R the Ka was found to be 9.3×10^{12} M^{-1} and the capacity 4.26×10^{-11} moles/mg protein.

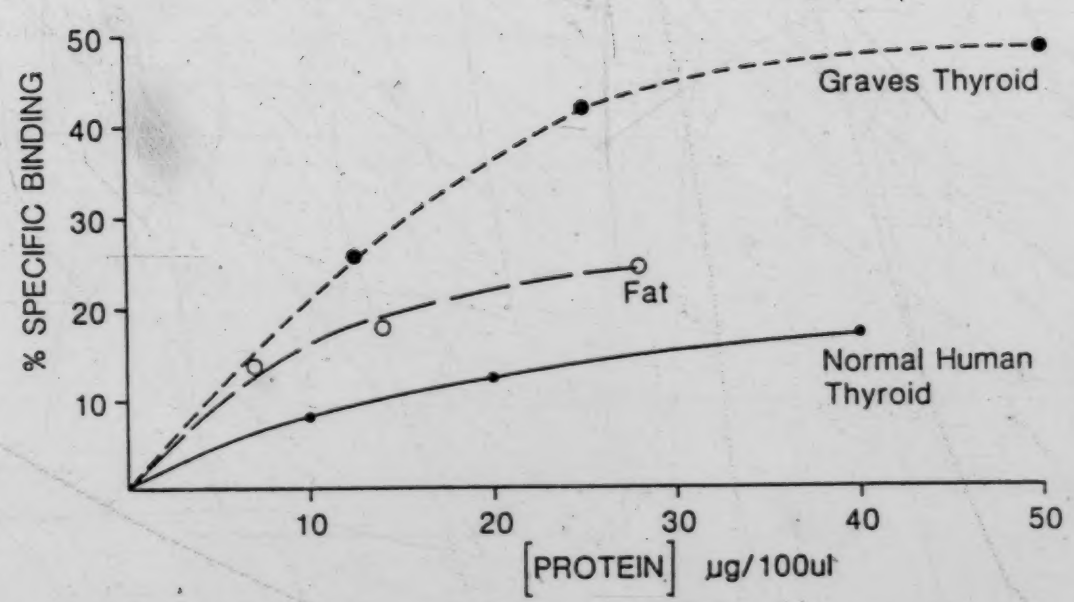


Figure 7. Binding of ^{125}I-TSH to various TSH-R preparations. Comparison of ^{125}I-TSH specific binding to increasing protein concentrations of TSH-R preparations made from human thyroid (Graves' or normal) and guinea pig fat cell membranes. Identical binding conditions were used for the three preparations. Non-specific binding, obtained for each protein concentration by addition of excess cold TSH (10^5 fold) was subtracted from the maximum binding.

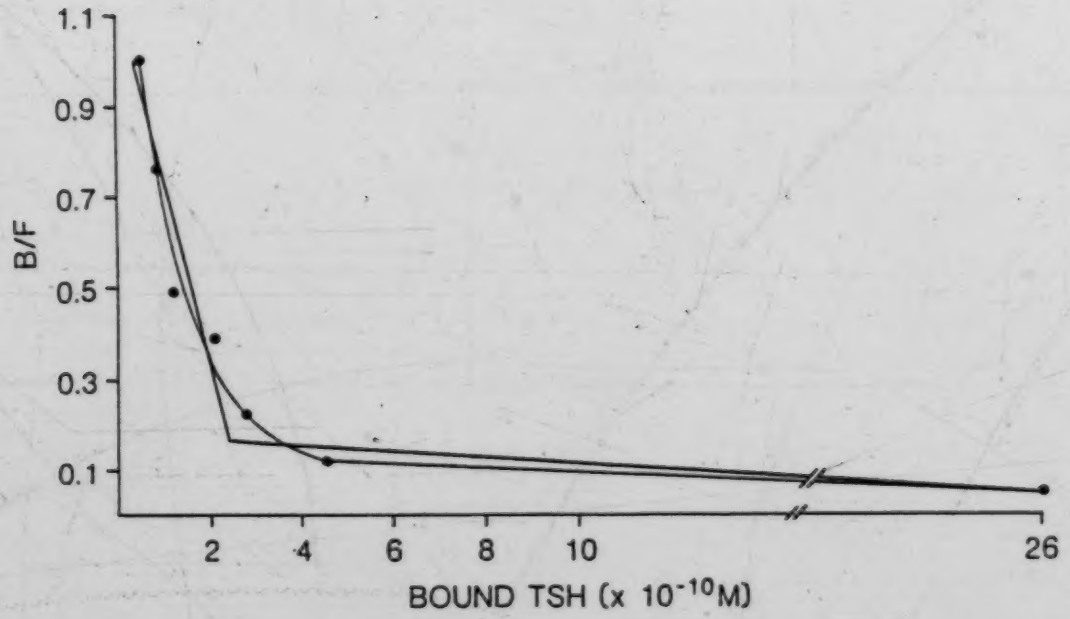


Figure 8. Scatchard plots of ^{125}I-TSH binding to solubilized Graves' thyroid TSH-R. Increasing concentrations of cold TSH as in the particulate thyroid membrane study were used. Cold and ^{125}I-TSH were incubated with the TSH-R preparations at 37^{0}C for 15 min.

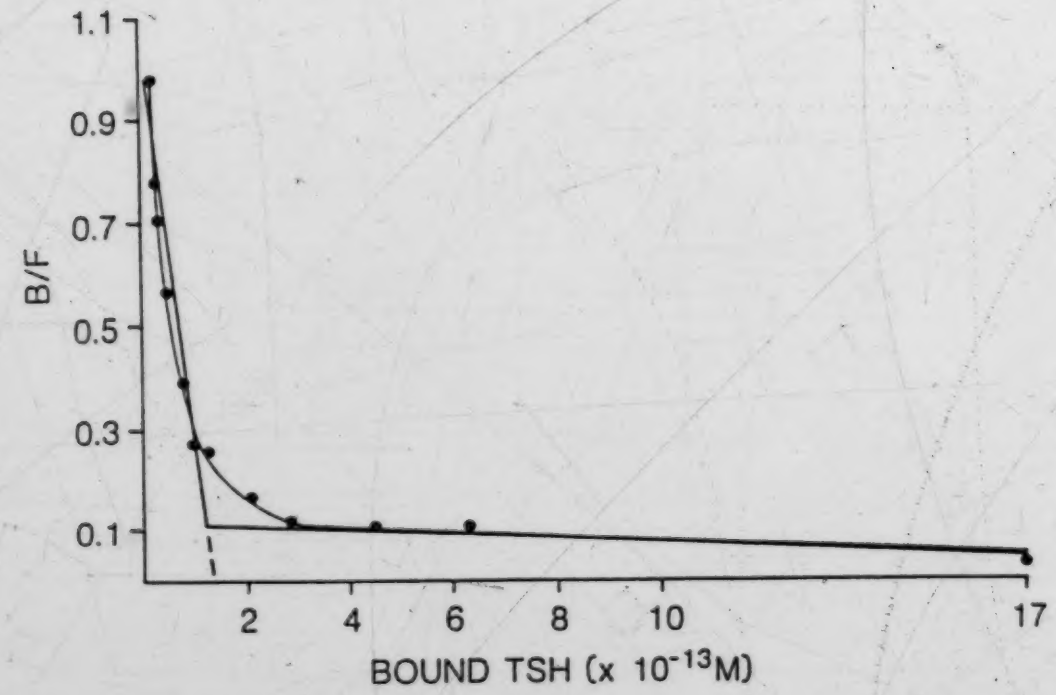


Figure 9. Scatchard plot of ^{125}I-TSH to solubilized guinea pig fat cell membranes.

b) TSH-R Purification

i) TSH-Affinity Chromatography Columns

The coupling efficiency of commercial bTSH (Thytropar or Harris) to Affi-gel 10 was calculated in 12 experiments using 50,000 cpm of purified ^{125}I-bTSH as a marker and in 1 experiment using large amounts (1.2×10^9 cpm) of the same material. In the former, 85 to 99% of the radioactivity was found to covalently bind to the agarose gel. In the last experiment, 0.014% of the radioactivity could be recovered in the eluate.

To evaluate detachment of the ligand TSH from the agarose bed during the TSH-R purification procedure, determination of non-labelled TSH concentration in the eluate was done by RIA (Table 1). TSH could not be detected in the first eluate. In the second eluate (3 M NaCl in 25 mM Tris) the concentration was 2.2 µU/ml. For every column preparation 100 U of b-TSH was used and the final volume of the concentrated eluate was 2 ml giving a detachment of bTSH of 4.4×10^{-6} U in the eluate.

When the coupled gel was allowed to dry even minimally, over 25% of the radioactivity could be recovered in the eluate.

Columns were stored in 25 mM Tris at 4°C in presence of 0.02% of sodium azide and could be used for purification of the TSH-R up to 12 times over periods of at least 3 months.

Table 1

Characteristics of Partially Purified TSH-R Preparation

	Thyroglobulin (ng/ml)	TSH (μU/ml)	^{125}I-TSH Binding* (dpm/μg protein)
Crude membrane preparation	189×10^4	-	140
Solubilized TSH-R	-	-	117
Eluate buffer A	297×10^3	none detected	48
Eluate 3M NaCl	584	2.2	3140

*Results obtained with 1 μg of protein in the assays. Specific binding of ^{125}I-TSH to 0.02 μg of partially purified TSH-R preparation was 400 dpm. Thus, when the binding assays were performed using total protein content on the range that binding of ^{125}I-TSH had not reached saturation (for either TSH-R preparation), the calculated degree of TSH-R purification was approximately 143 fold, as compared to the binding using particulate membrane preparation.

ii) Affinity Chromatography Purification of the TSH-R

Solubilized TSH-R preparations obtained from normal or Graves' thyroid tissue were applied to TSH affinity chromatography columns for purification of the TSH-R. After 12-16 hours of incubation at 4°C elution and extensive washing of the non-absorbed material, the bound portion was eluted with 3 M NaCl buffer as seen in Figure 10.

The 3 M NaCl eluate had to be dialyzed and concentrated prior to its use in the binding assays. In the first experiments samples were concentrated in Minicon concentrators, but as a portion of the protein was lost on the membrane filter and the plastic container, samples were later concentrated under vacuum at 4°C during dialysis.

Protein content of the purified TSH-R preparation was usually from 2 to 10 µg of protein/100 µl. Since these values are in the lower range of sensitivity of the Bio-Rad protein determination (micromethod) they are probably not accurate and the real concentration was even lower than what was read. Also, it was later observed that the initial concentration of detergent present in the 3 M NaCl eluate (0.05%) increased with the sample concentrating procedure to at least twice the original value and since the protein concentrations in the samples were extremely low, the contribution of the detergent to the absorbance reading of the protein was probably higher than expected. However, since it was not possible to re-estimate the protein concentrations of samples prepared and used before, no correction of the values was made.

^{125}I-TSH binding to the purified TSH-R preparation was measured and compared to other preparations to establish the degree of purifi-

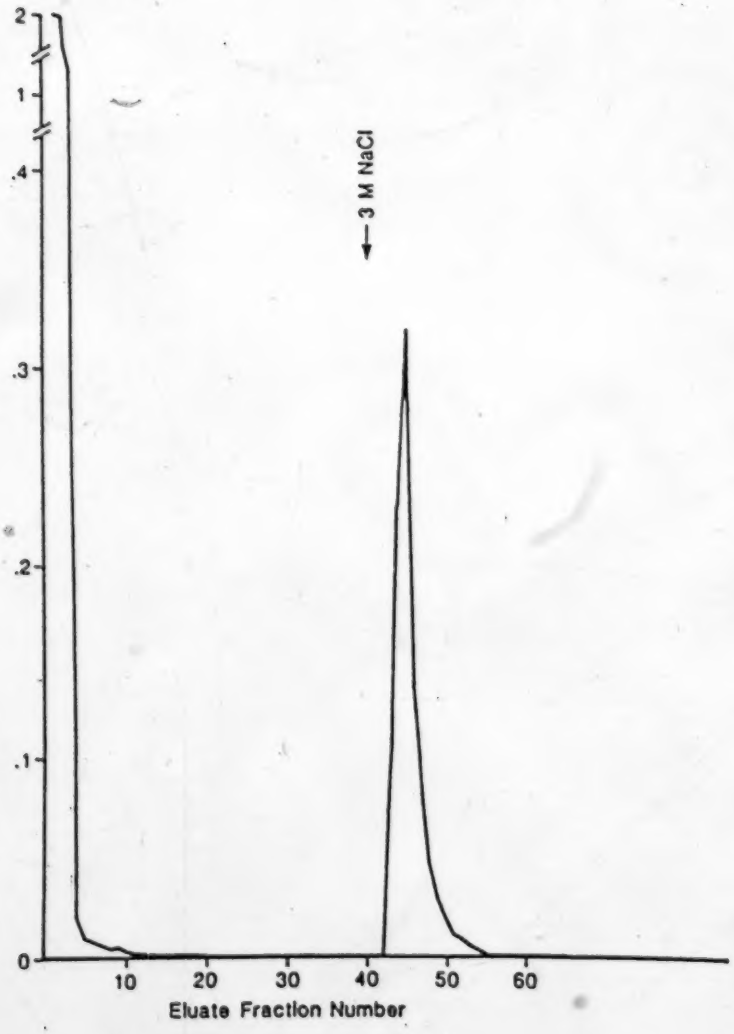


Figure 10. Eluates from TSH affinity chromatography column were monitored by spectrophotometric absorption. Elution of bound TSH-R was done with 25 mM Tris solution pH 7.4 containing 0.05% Triton and 3 M NaCl. Fractions were pooled, dialyzed and concentrated prior to storage. TSH-R purification procedure by this method required a minimum of 32 h.

cation. Thyroglobulin and TSH content were measured in the different samples (Table 1). For the above mentioned problems with determination of the protein concentrations, the degree of purification in terms of ^{125}I-TSH binding is higher than what the Table 1 results show. Although the concentration of Tg was reduced by more than 3,000 times it was still present in the purified TSH-R preparation. Also, the presence of TSH in this preparation might have interfered with the binding of ^{125}I-TSH giving a low estimate of the degree of purification.

Scatchard analysis of the binding data (Fig. 11) revealed the presence of 2 binding sites, of high and low affinity, as in the solubilized non-purified preparations of the TSH-R. The Ka for the high affinity binding sites was $4.3 \times 10^{9}\ M^{-1}$.

c) Characterization of the Purified TSH-R

i) Electrophoretic Analysis

Disc gel electrophoresis in 7.5% polyacrylamide and Coomassie blue staining revealed the presence of 2 protein bands. When affinity chromatography-purified TSH-R was analyzed in 10% slab gel electrophoresis under dissociating conditions (SDS-PAGE) and gels stained with silverstain, at least 8 bands were distinguished. One band with molecular weight of 88,500 was enhanced with respect to the solubilized non-purified electrophoretic profile.

The TSH-R-^{125}I-TSH complex analyzed in disc gel electrophoresis (under non-dissociating conditions) revealed the presence of a radio-

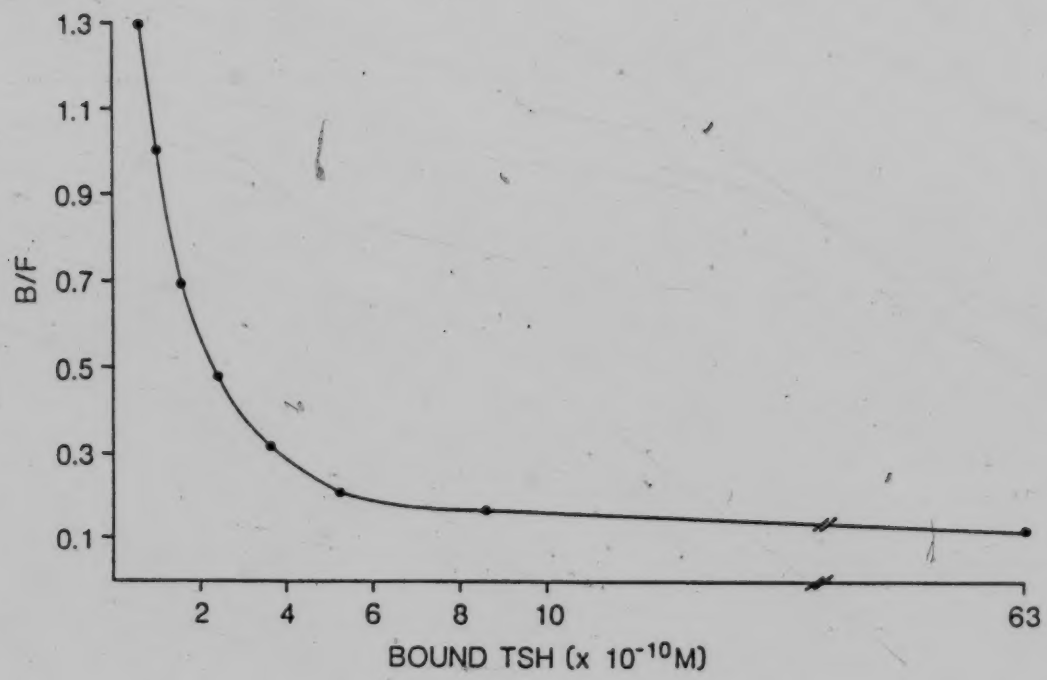


Figure 11. Scatchard plot of specific ^{125}I-TSH binding to affinity chromatography purified TSH-R. Assay conditions were the same as used for solubilized non-purified TSH-R preparations.

activity peak at the same localization as standard protein aldolase, which was not present when ^{125}I-TSH was electrophoresed alone (Fig. 12).

ii) Continuous Sucrose Gradient Sedimentation

Analysis of purified TSH-R preparation by sucrose gradients showed that the maximum specific binding capacity was localized between 4.15 and 4.9 S as seen in Fig. 13.

The TSH-R-^{125}I-TSH complex had the same sedimentation coefficient as aldolase, suggesting a complex molecular weight of 150,000 (Fig. 14). A major peak was found at the same localization as ^{125}I-TSH alone. The presence of this major peak suggests that most of the bound ^{125}I-TSH becomes free during the procedure, which takes more than 16h.

d) Human IgG Immunoabsorbent Columns

Since by TSH affinity-chromatography it was not possible to obtain a pure preparation of TSH-R, and knowing that IgG's from patients with Graves' disease inhibit the binding of ^{125}I-TSH to the receptor, presumably by binding to the receptor itself, immunoabsorbent columns were prepared using DEAE-purified IgG from a patient with Hashimoto's thyroiditis (column 1) and with IgG from a patient with Graves' disease (column 2) as described in the Methods section.

Coupling efficiency of IgG to the CnBr Sepharose 4B gel was 95 to 99%. Triton N-101 solubilized human TSH-R preparation was applied to

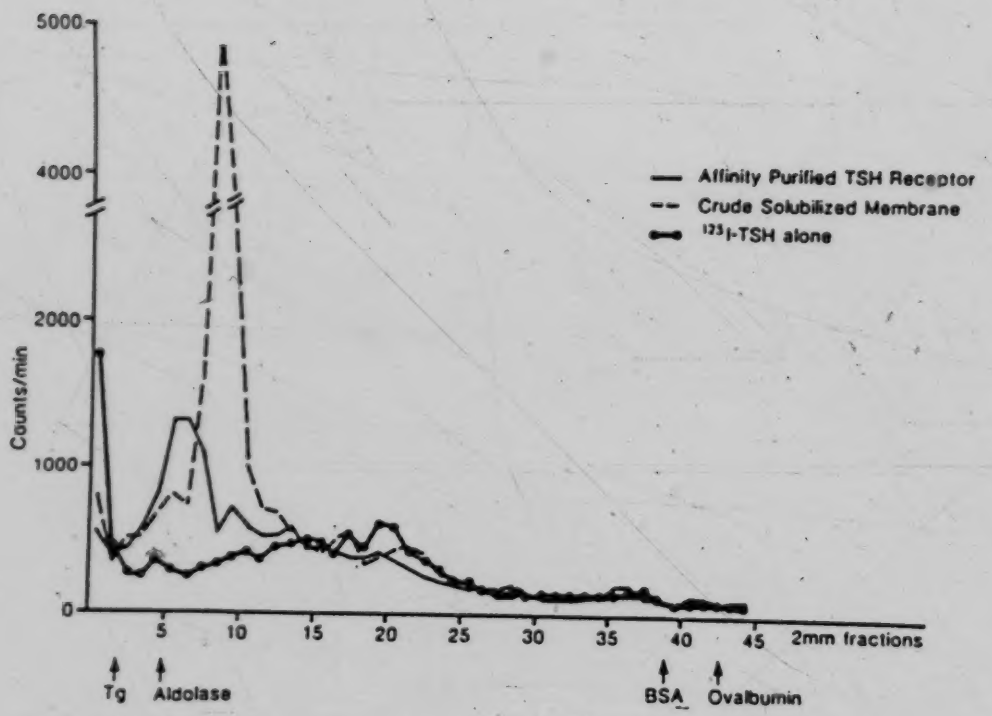


Figure 12. Radioactivity profile of 7.5% polyacrylamide disc gel electrophoresis of the TSH-R - ^{125}I-TSH complex. Preparations of purified TSH-R were incubated with ^{125}I-TSH for 1 h at 24^{o}C prior to application to the disc gels. Gels were sliced into 2 mm fractions and radioactivity determined.

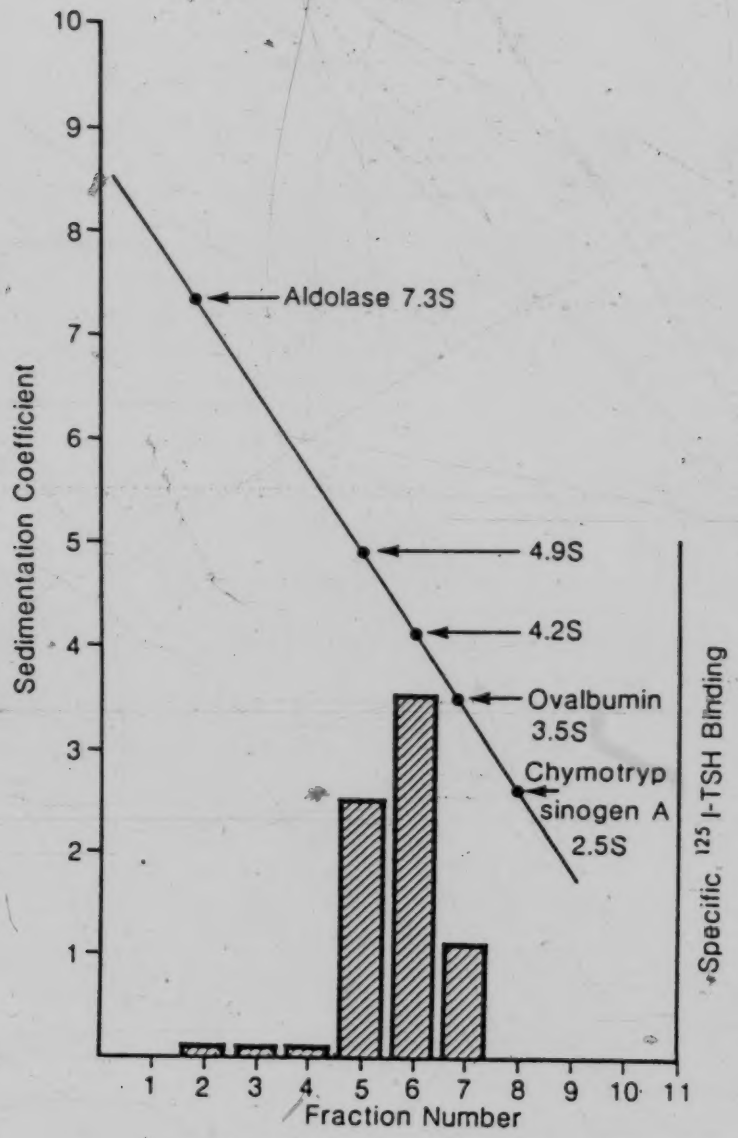


Figure 13. Sucrose gradient analysis of purified TSH-R. Receptor samples (100 µl) in 25 mM Tris, 0.05% Triton X-100 were applied to 4 ml sucrose gradients (8-25% w/v). Sucrose gradients were centrifuged for 14h at 4°C at 230,000 x g. Fractions were tested for their capacity to specifically bind ^{125}I-TSH.

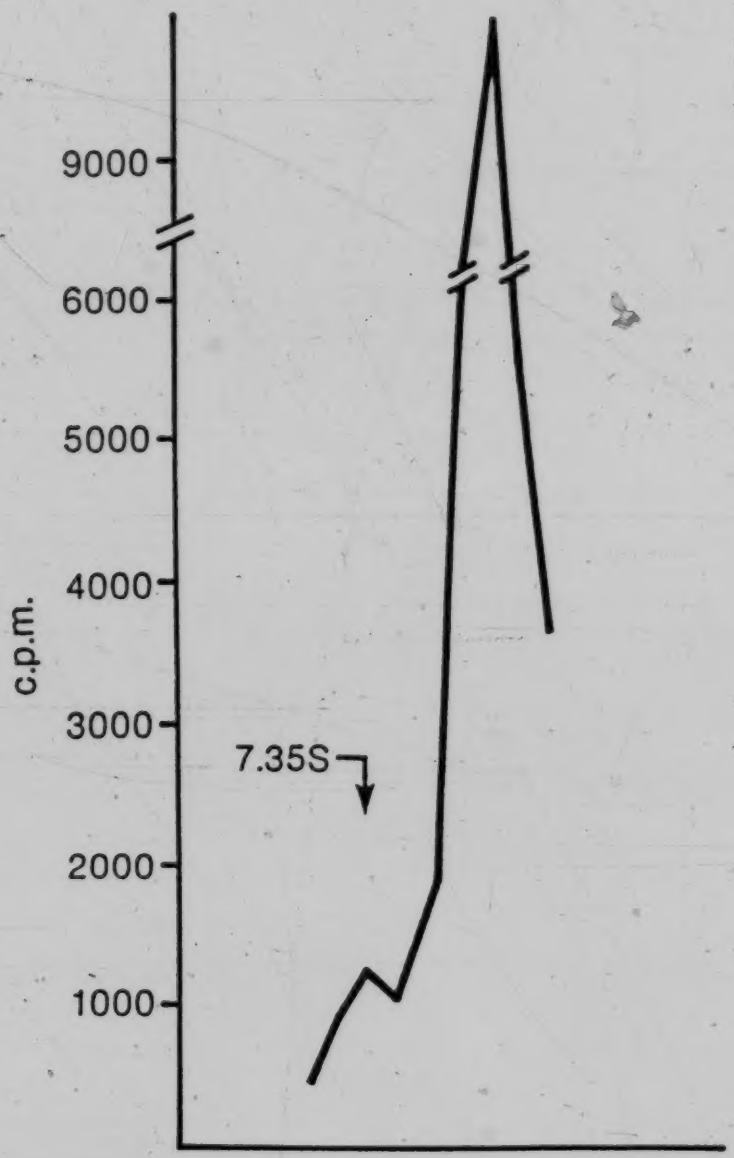


Figure 14. Radioactivity profile of ^{125}I-TSH - TSH-R complex sedimented in sucrose gradient. Labelled hormone and TSH-R were incubated for 1 h at 24^{o}C prior to the sedimentation procedure. A control gradient containing ^{125}I-TSH alone, showed a single absence of the radioactive peak at 7.35 S.

both columns, and eluates (Tris/Triton - eluate 1 and Tris/Triton/NaCl - eluate 2) from each were applied to the other column, as seen in Table 2. Protein concentrations obtained in the different samples were comparable, or else were matched by dilution before the binding assay was done. Binding assay results are summarized in Table 3. Eluate 2 from column 1 and eluates 1 and 2 from column 2 (both in phase I) were studied by electrophoresis (10% SDS-PAGE) and stained with silver. In Fig. 15, clear enhancement of several bands can be seen. Eluate 2 from column 1 shows the same pattern as the control sample. Eluate 1 from column 2 has several low molecular weight bands enhanced with respect to control, and eluate 2 from the same shows a clear enhancement of 2 bands at approximately m.w. of 50,000 and 45,000. Higher molecular weight proteins, although possibly present, could not be detected.

Thyroglobulin determinations demonstrated that eluates with 3M NaCl (2nd eluate) from both columns had high degrees of Tg impurity (1090 ng/ml and 780 ng/ml, immunoabsorbents 1 and 2 respectively).

Thyroglobulin was also detectable in the eluate 1 from column 2, with a concentration of 590 ng/ml. However when columns were washed with glycine solution, pH 2.8 after eluate 2 the thyroglobulin concentration in the former was negligible.

3. DISCUSSION

Of the several reported methods to obtain particulate TSH-R preparations (Wolff et al. 1971, Smith et al. 1974, Mehdi et al. 1975), membranes purified in sucrose gradients and crude membranes

Table 2: TSH-R Purification by human IgG immunoabsorbents.

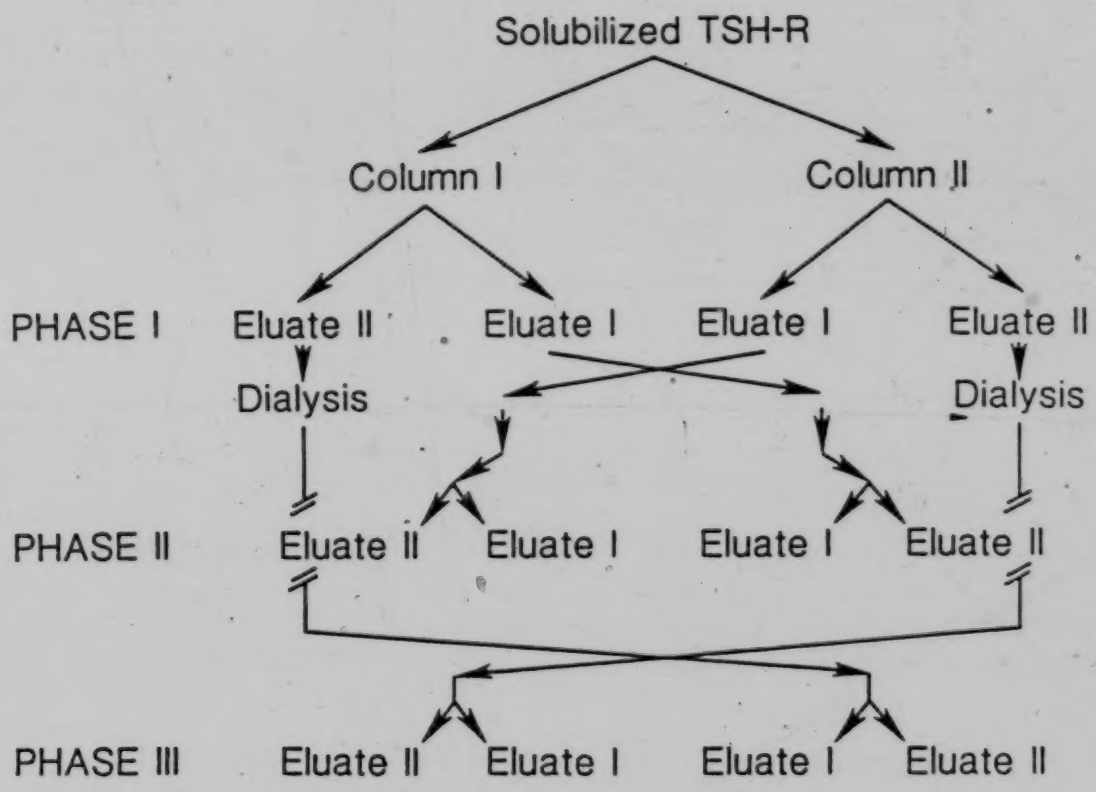


Table 3

^{125}I-TSH Specific Binding (%) to Immunoabsorbent Columns' Eluates

	COLUMN 1		COLUMN 2	
	ELUATE I	ELUATE II	ELUATE I	ELUATE II
Phase I	16.1	3.3	7.2	2.4
Phase II	.7	1.5	0	.9
Phase III	.7	2.6	1.7	2.1

Results are expressed as percentage of specific binding. Binding of control sample (solubilized, not applied to any column) was 51.2%. This sample had a protein concentration 10 times higher than the concentrations obtained in eluate I from either column.

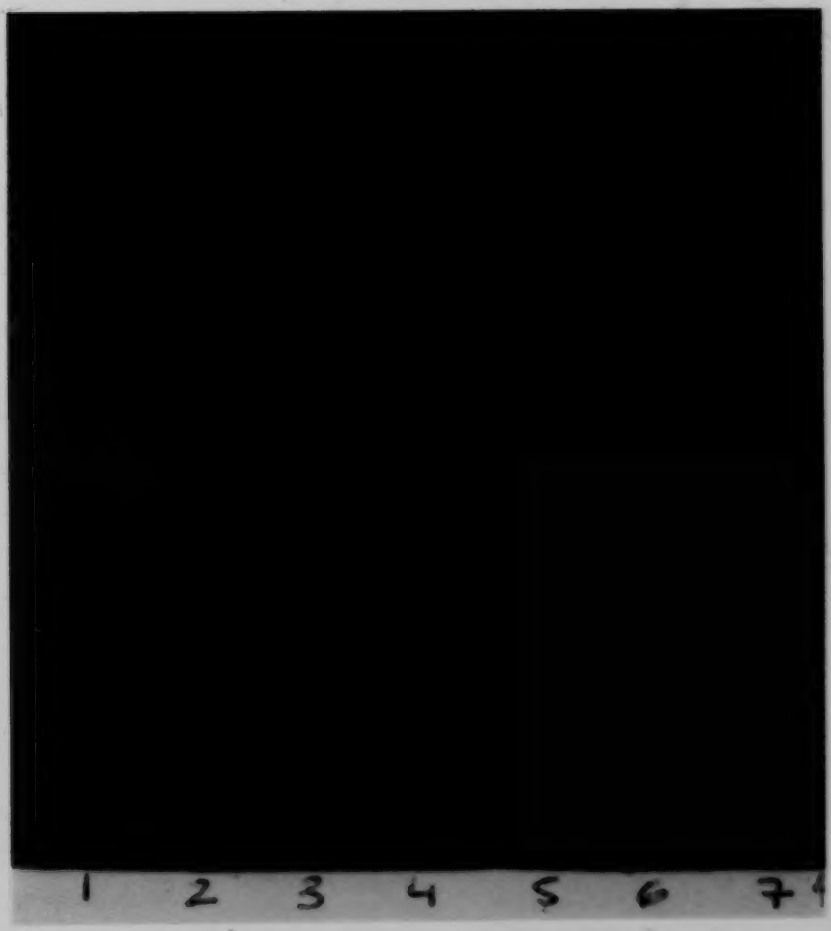
Figure 15. Electrophoretic profile of eluate from human IgG immunoabsorbent columns.

Line 1: Control sample

Line 2-3 Eluate 2 from immunoabsorbent column 1

Line 4-5 Eluate 1 from immunoabsorbent column 2

Line 6-7 Eluate 2 from immunoabsorbent column 2


1 2 3 4 5 6 7

were studied. The easiness of preparing the latter, as well as the yield of TSH-R obtained made it more suitable for large scale TSH-R purification. It is also important to note that no specific binding of ^{125}I-TSH to intracellular organelles has been reported which favours the use of crude particulate membrane preparations (Mehdi et al. 1977).

Beall et al. (1979) reported that iodination of TSH could interfere with binding of the labelled hormone to the TSH-R. They found that, by the Bolton-Hunter (1973) acylation method, binding was prevented whereas iodination by the chloramine-T method (Greenwood et al. 1963) resulted in good binding activity. Lactoperoxidase has been found to give good iodination of different protein molecules and is presently used in many laboratories for the labelling of purified b-TSH (Silverberg et al. 1978, Pekonen et al. 1980, Ozawa et al. 1979a, Koizumi et al. 1982).

Although some authors have found that the best maximum binding of ^{125}I-TSH to TSH preparations was found at low pH (5.5) (Tate et al. 1975), it was preferred to maintain a more physiological pH in the binding assay with which good reproducible binding was found at low protein concentrations (20-50 µg/100 µl). Preparations were standardized for their capacity to bind ^{125}I-TSH in the absence of salt media and in the presence of 50 mM NaCl (for their utilization in the TBI assay) since different salt concentrations vary the degree of binding to the labelled hormone (Pekonen et al. 1979).

The affinity constants and binding capacities found in the standard TSH-R preparations used (particulate and solubilized) revealed the presence of a high affinity binding site with a Ka similar to the

values found by others (Pekonen et al. 1979, Koizumi et al. 1982). However, other authors have found only one high affinity binding site (de Bruin et al. 1982a) on Scatchard analysis of the data obtained from bindings performed at 25°C, and a curvilinear Scatchard plot with similar affinity constant when the binding assays were performed at 37°C (de Bruin et al. 1982b) or at 0°C for 18-24 h (Petersen et al. 1977) or at 37°C for 10 min or 2 h (Rees Smith et al. 1977).

Although no proteinase inhibitor was included in the buffers, samples were stable with respect to ^{125}I-TSH binding even for months, provided that they were quickly frozen at -70°C and used only once (not re-frozen). This was particularly important for the particulate membrane preparations, otherwise, within 6-8 h at 24°C maximum specific binding could decline by 25-30%.

The affinity chromatography method described provided a purification of the solubilized TSH-R by a factor of 140 as judged by the binding to ^{125}I-TSH. The Affi-gel 10 agarose (an N-hydroxysuccinimide ester) proved to be feasible for the coupling of cold hormone as seen by the coupling efficiency calculations and measurement of TSH by RIA in the eluates. The columns were stable at 4°C and could be used several times, allowing the purification of TSH-R from large samples. Thus, enough purified receptor to be used in further studies, such as characterization and production of monoclonal antibodies, could be obtained. Receptors from more than 500 g tissue equivalent were purified by this method.

Purified TSH-R also gave a curvilinear Scatchard plot as has been found by others (Koizumi et al. 1982). The finding of a curvilinear Scatchard plot on binding of labelled hormones to receptor has

previously been described for several polypeptide hormone receptors, but no clear explanation for it has been found. De Meyts et al. (1976) proposed that the curvilinear Scatchard plots found on the binding of ^{125}I-Insulin to its receptor resulted from a negative cooperativity site-site interaction among one homogeneous class of receptors. The negative cooperativity is supposed to be the consequence of ligand-induced interaction of receptors or receptor subunits, by which a decrease affinity of the receptor for the ligand appears as increasing number of receptors are occupied. However, as indicated by Powell-Jones et al. (1979, 1980) this explanation is not valid for the TSH receptors, since the rate of dissociation of ligand from TSH-R is independent of the binding site occupancy over a 40 fold range, corresponding to a 100-fold range of free TSH concentration and only the high affinity binding sites are destroyed by heating the membranes at 50^{o}C for 20 min prior to the binding assay. They concluded that ^{125}I-TSH binds to heterogeneous binding sites of which probably only the high affinity class are TSH-R. The same authors (Rickards et al. 1981) found only one high affinity binding site in purified and crude solubilized TSH-R preparations containing 1% or 15% detergent (Lubrol) in bindings carried out at 37^{o}C fo 1 h. The differences in detergent concentrations as well as the length of incubation (1 h vs. 15 min) might explain the discrepant results since it was previously observed that the presence of high detergent concentrations partially inhibited ^{125}I-TSH binding. Similarly, it was previously observed (unpublished data) that with solubilized TSH-R preparations, binding of ^{125}I-TSH is maximum at 15 min (at 37^{o}C) and it decreases thereafter, increasing the non-specific binding.

From the studies of measurements of Tg and TSH it can be concluded that, although a certain degree of purification was achieved, the TSH-R preparations were not pure. An indication of that was the finding of several electrophoretic bands detected by silverstain also implying that, by those methods it was not possible to identify the TSH-R. Again, the very property of the receptor (to specifically bind TSH) was used in an attempt to localize the studied molecule. Fractionation in sucrose gradients showed specific binding to fractions between 4.2 and 4.9 S. Interestingly this sedimentation coefficient is very close to the one found to absorb TSAb activity from Graves' IgG. Tate et al. (1975) had found, by similar methods, that ^{125}I-TSH could bind to 4 different fractions of the sucrose gradients. Since their preparation had 5 to 50 times higher detergent concentration, which is known to form micelles, the peaks found at 280,000 and 160,000 M.W. could well be the result of the detergent concentration in the samples studied. Similarly, Dawes et al. (1978), reported a high molecular weight component (300,000 of the TSH receptor, separated by Sepharose chromatography. However, they also found the same M.W. for the complex ^{125}I-TSH-TSH-R eluted in 0.5% detergent and this was also the localization found for the same concentration of detergent eluted alone in absence of receptor preparation and or labelled TSH. These results would suggest that the molecular weights found for the receptor are the result of the micelles formed by the detergent present in the solubilized preparations.

When the complex ^{125}I-TSH-TSH-R was analyzed by sucrose gradient sedimentation and electrophoresis under non-dissociating conditions it was localized at the same position as aldolase, used as standard

protein, indicating a complex M.W. of 150,000. On the electrophoretic profile of TSH-R under dissociating conditions and detected by silver-stain, only the band of 88,500 M.W. was enhanced with respect to the solubilized preparation. This band, also found in the purified material of Koizumi et al. (1982) could be the TSH-R or a fragment of it. In the former case, the ^{125}I-TSH-TSH-R complex M.W. of 150,000 could indicate that the TSH-R would bind 2 molecules of TSH simultaneously.

Since the purpose of the purification procedure was mainly to achieve a TSH-R preparation devoid of other antigenically important thyroid molecules, Tg determinations were necessary. Although a 3000-fold decrease in Tg was obtained, Tg was still present in considerable concentrations in the purified TSH-R preparation. Although the large M.W. of Tg could facilitate its separation from the TSH-R by standard chromatography, this approach was not elected for several reasons. First, the TSH-R M.W. was not known with certainty and could (because of aggregates or detergent micelle formation) elute close to Tg and, secondly, because fragments of Tg could also migrate with the TSH-R the assumption could not be made that it would be possible to differentiate both molecules on the basis of M.W. alone. Since IgG from patients with Hashimoto's thyroiditis containing Tg Ab devoid of TSI activity were available, as well as purified IgG from Graves' patients, the immunoabsorbent chromatography procedure was preferred and undertaken.

Although preliminary studies showed that the eluate of non-absorbed material from column 1, absorbed in column 2 and eluted with high salt concentrations was enriched on ^{125}I-TSH binding activity, when the studies were repeated and eluates from both columns cross-

absorbed, no difference in binding capacity was found between the high salt eluate from both columns. Thus, the approach did not prove to be useful for the TSH-R purification. Moreover, Tg concentrations in the different eluates were even higher than those obtained by affinity chromatography with TSH.

The finding that it was impossible to purify the TSH-R by using Graves' IgG (previously shown to inhibit ^{125}I-TSH binding to particulate and solubilized TSH-R preparations and stimulate cAMP production in human thyroid slices and rat thyroid cells) indicated that either the IgG binding site is not exactly the same as the TSH-R or that dissociation rapidly takes place under the mild conditions used (20 mM Tris, 0.05% Triton N-101).

CHAPTER IV

MONOCLONAL ANTIBODIES

1. INTRODUCTION

Since Kohler and Milstein (1975) first described the production of monoclonal antibodies (MA) such antibodies have been used for a wide variety of applications of which the identification of antigens has been one of the most important. Since the antigenic site of the Graves' IgG is thought to be the thyroid TSH-R, it was of much interest to develop MA against the TSH-R. The purpose of that project was twofold. With the availability of MA it would be possible to purify the antigen by means of immunoabsorbance as described by Secher and Burke (1980), in their successful purification of human interferon. With a pure antigen it would be possible to study the cell-mediated immune response of patients with Graves' disease. Also, by producing different MAs of known antigenic specifity, the response of Graves' IgG and its site of interaction with the thyroid cell could be explored, as well as their mechanism of action and IgG production by patients with this disease.

The selection of those clones producing a desired specific antibody is one of the main problems encountered when the immunizing antigen is not pure. Our preparation of partially purified TSH-R by affinity chromatography, although 140 times more pure than the solu-

bilized non-purified preparation still contained several other molecules apparently unrelated to the TSH-R. Thus it was necessary to develop a screening assay that could differentiate the TSH-R from other potential antigens in the preparation. The only known function of the TSH-R that could be used in these assays was the binding of the receptor to ^{125}I-TSH since it is fast and allows the testing of many samples in a short time.

High affinity TSH-R have also been found in guinea pig fat cell membranes (FCM) (Teng et al. 1975, and Kishihara, et al. 1979), which have been used for purification of Graves' IgG (Endo et al. 1981). They offer the advantage of not reacting with either thyroglobulin or microsomal antibodies.

For the above reasons, MA against normal and Graves' human thyroid as well as against guinea pig fat cell membranes were produced and the antigenic specificity of several antibodies was studied.

2. RESULTS

The mouse immunization procedure has been previously described in the Methods section. At least 3 injections were given to every animal prior to testing for their immunological response in terms of IgG production to the antigen injected. Animals that responded positively to the ^{125}I-sheep anti-mouse IgG (SAM) or to the TBI assays using FCM and ^{125}I-TSH were used for the fusions following a fourth boost 3 days prior to being sacrificed. A total of 11 fusions were done, 4 mice were immunized with antigens prepared from Graves' thyroid, 6 were

normal thyroid antigens and 1 used an antigen prepared from guinea pig fat cell membranes. Of these, only 4 fusions resulted in clear positive clones (3 arose from normal human thyroid TSH-R preparations and 1 from FCM). From the remaining fusions, some clones either did not survive or became contaminated while, from other healthy clones, no positive antibodies could be detected by the screening assays used.

a) Positive Fusions

i. Fusion #1 (from normal thyroid TSH-R preparation). Fused cells were distributed in 32 plates of 96 wells each. Ten days after the fusion, clones were macro or microscopically visible in most of the wells (approx. 80% of them). The first screening assay was done by pooling media from 4 consecutive wells into 1 testing well, which had been previously coated with 50 µl of 50 µg/ml normal thyroid TSH-R purified by affinity chromatography and from which the excess detergent had been removed.

Of the 2,016 wells screened in the first test, 188 were tested individually and 43 were found to have clones secreting IgG against the partially purified TSH-R preparation. Since most of the wells contained more than one clone, limiting dilutions were carried out to obtain single

positive monoclones. The original clones and the ones grown after limiting dilutions were tested an additional two or three times, finally obtaining 21 monoclones.

The screening assays (^{125}I-SAM) used in this fusion detected antibodies against all the antigens in the testing preparation not only against the TSH-R.

In a previous fusion the possibility of using the TBI assay in test tubes for screening of the medium had been investigated, but medium alone, without any antibodies, inhibited the binding of ^{125}I-TSH to the human thyroid TSH-R preparations. Thus it was necessary to produce large amounts of MA in order to extract them from the medium. Unfortunately, of the 21 monoclones selected, 17 became contaminated shortly before a sufficient number of cells could be harvested for injection into irradiated mice for ascites production. The other 4 clones were used in part for ascites production while the remainder were kept frozen for later use. IgG from ascites fluid was purified by ammonium sulphate precipitation and tested in the regular TBI assay. Although they were positive against the whole antigen, none of the 4 were able to inhibit the binding of ^{125}I-

TSH to solubilized human thyroid TSH-R preparations.

These MA however, were tested for their ability to stimulate the production of cAMP in human thyroid slices and only one, at high concentration (2.5 mg/ml) was minimally positive (150% over control) and when tested for a second time, was negative.

ii. Fusion #2 (FCM immunized mice) To avoid unnecessary use of antigen preparation, clones were first selected for the presence of IgG in the media. Of the 74 clones that produced IgG, 25 produced antibodies against the antigens injected. These clones were then tested in the TBI assay using particulate FCM in polyvinylchloride plates (PVC plates), but none were found to significantly inhibit the binding of ^{125}I-TSH to the membrane preparation. However, among the 25 positive clones for IgG against FCM, 7 strong positives were selected and grown in culture.

The medium was concentrated under vacuum and dialyzed against saline to achieve a more concentrated solution of MA, which was then retested in the TBI assay. Although samples were concentrated over 10 times, no MA giving a positive TBI were found.

iii. Fusion #3 (from mice immunized with normal human thyroid.) 34 clones were found to produce IgG against the antigen used to immunize the mice, of which 3 were also positive for FCM antigens. However, after testing several times, 18 clones were finally selected, the others having become negative. Of these 18 clones, 7 produced a significant ^{125}I-TSH binding inhibition when tested in the FCM-TBI assay in PVC wells. Although the assay reproducibility was very good when purified IgGs were used, in presence of cell medium it was variable. Hence, it was repeated several times and only 2 were found to inhibit the binding of ^{125}I-TSH consistently . Some of the positive IgG in the previous assays not only did not inhibit binding of ^{125}I-TSH in subsequent assays, but resulted in an increase of up to 120%.
These clones however, were kept and the harvested cells were injected into mice. The IgG purified from the resulting ascites fluid was retested and found to be negative in the regular TBI assay (i.e. no binding inhibition or enhancement found). In the cAMP production assay 3 MA were found to increase the cAMP production of FRTL cells.

iv. Fusion #4 (from mice immunized with normal human thyroid.) Of 15 clones that were selected and tested for their TBI activity in an identical procedure as for fusion #3, 3 clones were found to be positive. Upon purification and retesting of the MA in the TBI assay, the results were negative.

b) Screening Assays

Since the sample preparations used to immunize the mice contained, in addition to the TSH-R, many other antigenic components which gave rise to a wide variety of monoclonal antibodies, attempts were made to develop a sensitive and specific screening assay.

Yavin et al. (1981) were able to develop MA against the bovine TSH-R. Their screening assay involved the use of poly-L-lysine to bind the TSH-R preparations to the PVC wells. However, since I was unable to obtain binding of ^{125}I-TSH to the receptor preparations by following their assay as described, different conditions were studied (variable protein concentrations and ligand, different incubation conditions in terms of time and temperature, and different buffers at variable pH), but without success. Binding of labelled TSH was not higher than 2-3% of the total added and these values did not allow one to differentiate specific inhibition of binding by IgG due to the large variability of intra-assay results.

Microsomal and thyroglobulin antibodies do not bind to FCM, even though TSH and Graves' IgG bind specifically (Endo et al. 1981). On this basis the FCM-TBI assay in PVC wells developed by Dr. Zakarija,

was used to select the clones-producing IgG against the TSH-R (Methods Assay II, in monoclonal antibodies screening assay).

c) Monoclonal Antibodies Studies

None of the MA obtained were positive when tested against thyroglobulin or thyroid microsomes.

The 4 MA obtained from the first fusion were studied for their capacity to bind to the TSH-R at sites other than the ^{125}I-TSH binding site by immunoabsorbance; the eluates being evaluated by their capacity to bind ^{125}I-TSH on SDS-PAGE, and for thyroglobulin concentration.

IgG coupling efficiency, calculated by the E_{co} of the eluate, ranged from 79% to 93%.

Neither 2 M NaCl nor the acid eluates of the observed material exhibited specific binding to ^{125}I-TSH, and the binding activity could be recovered in the first eluate with Tris-Triton.

From electrophoretic analysis of the different eluates no proteins could be detected by Coomassie blue staining. With silverstain, several faint bands were seen in the acid eluates of every immunoabsorbent. None of the bands appeared to be enhanced with respect to the control samples, although the pattern was not identical in every acid eluate sample.

Thyroglobulin, measured by RIA was found in all eluates; Tg content of the 2 M NaCl eluates ranged from 3 to 20% of the Tg content of the control sample, whereas in the acid eluate it was only 0.6% of the control.

3. DISCUSSION

Although most of the fusions were successful in terms of the number and survival of clones, none could be selected for production of MA against the TSH-R by the assays tested.

Since all mice were positive with respect to the antigen and the IgGs produced by the clones of the last 3 were also positive in the TBI assay, the difficulty in obtaining MA against the TSH-R was probably due to the fusion and the methods of selecting the clones. Special effort was made to develop a TBI assay that would be able to screen many samples in a short period of time, but as seen from the results, not even the FCM-TBI assay was consistent. The fact that positive clones could become negative over a few days (sometimes the interval between screening assays was only 4-5 days) pointed to the possibility that some components of the medium interfered with the binding of ^{125}I-TSH to the receptor. Controls consisting of medium alone were always included, but this did not exclude the possibility of changes in the concentration of some medium components when cells were present. Alternatively, the inconsistency of results could have been due to variations in the other components of the assay, although this is unlikely since maximum binding and non-specific binding of ^{125}I-TSH were very consistent from assay to assay. Moreover, a known positive purified Graves' IgG was always included as a control and results were similar in all assays.

The only remaining possibility was to purify the IgG from every "positive" clone, and although it is a very time consuming procedure this was done in some cases. It was of interest however, to study the

possibility of having MA against thyroglobulin or thyroid microsomes, since patients with Graves' disease usually have circulating antibodies to these antigens. Although the 2 M NaCl eluates from the MA immunoabsorbants contained large amounts of thyroglobulin, it was probably the result of non-specific binding of this large molecule to the gel matrix as it was also found (although in lesser amounts) in the TSH affinity chromatography purification of the TSH-R. To assess this possibility, the MA were tested in the tanned red cell agglutination technique, which confirmed the above conclusion.

In the electrophoretic studies of the immunoabsorbants' eluate no specific protein enrichment could be detected. The protein content of the samples was lower than 1 µg/100µl in most of the cases, which makes them difficult to detect even by silverstain. On the other hand, the proteins of the thyroid membrane have not yet been identified, hence, observing enhancement of even one protein band in any of the eluates would not have provided more information.

Since the TSH-R is a high molecular weight intrinsic protein it might contain antigenic sites different from the TSH binding site. The possibility of having developed MA that could not be detected by any TBI assay (against sites of the TSH-R) was also studied by the immunoabsorbants. Unfortunately, no specific ^{125}I-TSH binding was found in either the 2 M NaCl or the acid eluates.

Even though some of the MA stimulated cAMP production in the FRTL cell model, it can not be concluded that they are specific for the TSH-R, as Kohn et al. (1983) have assumed. Other molecules such as prostaglandins and cholera toxin are also able to stimulate the production of cAMP by binding to sites other than at the TSH-R (see chapter VI).

CHAPTER V

GRAVES' IgG - THYROTROPIN BINDING INHIBITION EFFECT

1. INTRODUCTION

In 1956, Adams and Purves described the presence of an abnormal thyroid stimulator in the serum of patients with Graves' disease. The substance was identified as an immunoglobulin G (IgG) by Purves and Adams (1961) Kriss et al. (1964) and Meek et al. (1964). The methods for the detection of Graves' IgG in patients with this disease have been the focus of attention by many investigators. Among them, the contributions of McKenzie (1960 and 1976), Adams et al. (1967), Yamashita et al. (1972), Onaya et al. (1973) and Kendall-Taylor et al. (1980) deserve special attention. However, no single assay was developed that was positive in 100% of patients with Graves' disease.

Since Mehdi et al. (1973) described the *in vitro* inhibition of thyrotropin binding (TBI) to its thyroid receptor by IgG from patients with Graves' disease, the assay has been popularized by Smith et al. (1974) and attempts were made to identify the antigenic specificity of Graves' IgG, as well as to correlate the binding inhibition activity of the IgGs with the outcome of the disease (Mukhtar et al. 1975, O'Donnell et al. 1978, Fenzi et al. 1979a, Ozawa et al. 1979, Teng et al. 1980, Davies et al. 1980, McGregor et al. 1980). However, large

discrepancies were found and Gossage et al. (1981) even showed a variable capacity of inhibition of TSH binding to the patients' own and to other thyroid tissues. Attempts to correlate biological activity (cAMP production) and the TBI capacity of Graves' IgG, have also given discrepant results (McKenzie et al. 1978, Endo et al. 1978, Sugenoya et al. 1979, Ozawa et al. 1979, Arikawa et al. 1980). The differences could have been partly explained by the different sources of thyroid tissue (human, bovine, etc) and by the various methods used to prepare the TSH-R (Zakarija et al. 1978a and 1978b). For this reason, it was of interest to compare the TBI activity of Graves' and normal IgG in different receptor preparations made from the same thyroid tissue, as well as the effects of these IgGs in the TSH-R of cells of various animal species.

The TSH-R is comprised of a glycoprotein and a ganglioside (Meldolsi et al. 1977). Because of their hydrophilic properties, the carbohydrate moities should be located on the external surface of the cell membrane.

Carbohydrates have been implicated in receptor binding and with insulin action (Cuatrecasas et al. 1973c) as well as in the antigenic sites in the immune system (Smith et al. 1980). In patients with Graves' disease, antibodies against sialo GM_1 ganglioside have been detected (Sawada et al. 1980), however their relationship with the IgG responsible for the hyperfunction of the thyroid is unknown.

Studying the effects of monosaccharides on the TSH-R, Moore et al. (1976) observed that N-acetyl-neuraminic acid (NANA) increased the binding of ^{125}I-TSH to the receptor and subsequent treatment of thyroid cell membranes with neuraminidase resulted in the loss of hormone

binding. However, Amir et al. (1973) and Trokoudes et al. (1981) were not able to discern any specific effects of carbohydrates on TSH binding.

Although the TBI activity of Graves' IgG has been found in solubilized TSH-R preparations (Petersen et al. 1977, Richards et al. 1981, Kotulla et al. 1981, Koizumi et al. 1982) the specific binding site for Graves' IgG has not yet been evaluated and it remains controversial if the binding is to the same TSH-R. In order to further study any relationship between TSH and IgGs for receptor binding properties, the modulating effects of NANA were analyzed.

2. RESULTS

a) Comparison of Graves' IgG TBI in Crude and Purified Human Thyroid Membrane Preparations

The thyrotropin binding inhibition activity of 36 IgGs from patients with Graves' disease and 10 normal controls were studied using crude and purified human thyroid membranes (TMP) that had been prepared simultaneously from the same gland to avoid possible variations that may arise from different TSH-R sources. All assays were done in parallel, testing the same IgGs in both membrane preparations. ^{125}I-TSH and unlabelled TSH were from the same batch. The IgGs used were previously purified in Protein-A Sepharose affinity columns and protein concentrations adjusted to 12.5 mg/ml. In the binding assay, protein concentrations of the purified and crude mem-

brane preparations were 62.5 and 22.5 µg/100 µl respectively, in order to obtain a maximum specific binding of ^{125}I-TSH of approximately 20% in both TMPs.

TBI of normal IgGs in crude and purified preparations ranged from 8 to 44% and from 0 to 46%, respectively. The IgGs were divided into two groups, I and II, for convenience in the assays. Each group had 5 normal IgGs to determine the normal TBI value. A Graves' IgG was considered positive when its TBI exceeded the 95% confidence limit of the normal TBI for the same TSH-R preparation in each group (Mendenhall et al. 1976).

Of all Graves' IgGs, 66.7% and 80.6% were positive in crude and purified preparations respectively. When the patient's IgG were analyzed according to their clinical status (euthyroid, hypothyroid and hyperthyroid) and to the therapy received, the number of total positive IgG's for each subgroup was very similar in both TMP. These data are summarized in Table 4. Although both TMP gave similar degrees of positives, when individual IgG's were analyzed, wide differences were noted between the TBI values obtained in CM and PM and 47% of the IgGs studied showed a positive TBI in one preparation, but were negative in the other. Only one IgG was negative in both TMP. No correlation was found between the TBI values obtained in both TMP for each Graves' IgG ($r=0.044$) (Fig. 16). A consistent pattern of TBI values could not be found, i.e. no membrane preparation gave a trend of higher or lower TBI. Some of the highest IgG's TBI in one TMP were negative or only slightly positive in the other TMP. These differences were found among all subgroups of patients (according to the treatment received). Individual data are shown in Fig. 17.

Table 4

Percentage of positive TBI in crude (CM) and purified (PM) preparations

	Clinical Status				Treatment	
	Hyperthyroid	Euthyroid	Hypothyroid	None	^{131}I *	PTU •
CM	73.7	61.5	50.0	73.3	75.0	50.0
PM	79.0	84.6	75.0	73.3	90.0	75.0

* IgG samples from patients treated with ^{131}I were obtained 3 months after therapy was given.

• For the propylthiouracil (PTU) sub-group blood was drawn during treatment. Positive TBI were more frequently found in PM, mostly due to positive TBI among treated euthyroid and hypothyroid patients.

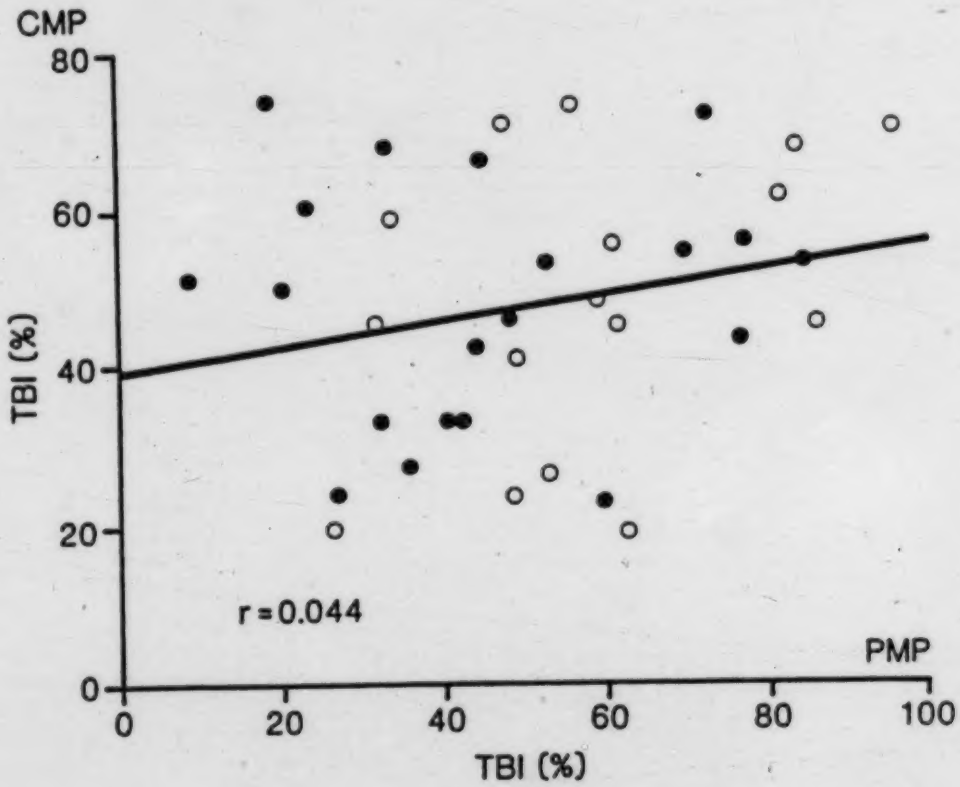


Figure 16. Comparison of TBI effects of purified Graves' IgG on crude and purified particulate human thyroid membrane preparations made from the same gland. Black circles, Group 1; open circles, Group II. (see text).

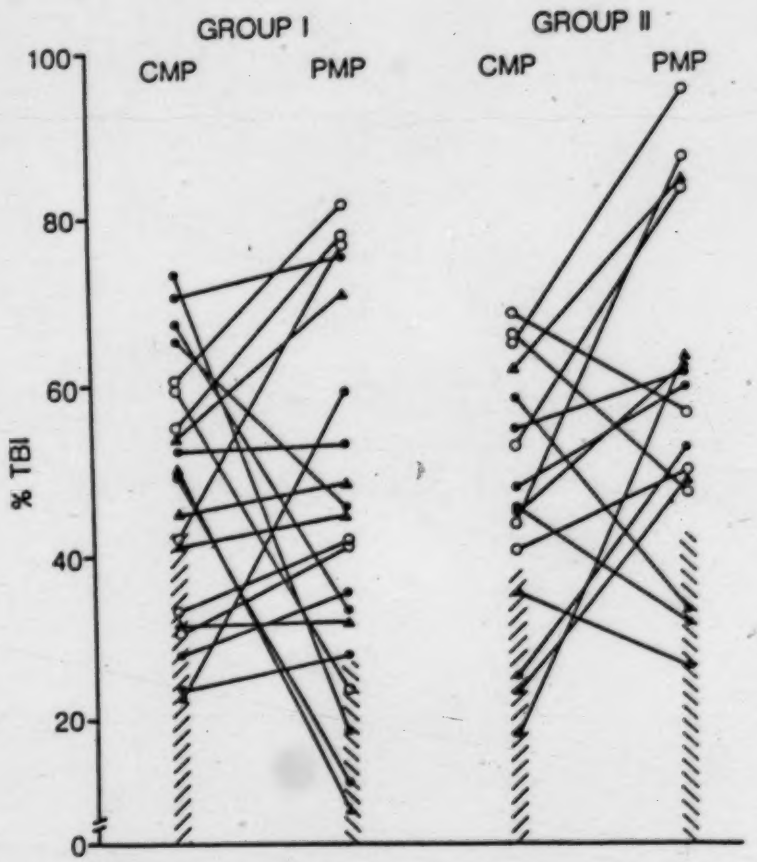


Figure 17. TBI values for each IgG in crude and purified thyroid membrane preparations. Shaded areas denote negative TBI. • non-treated patients, ▲PTU treated patients, o ^{131}I treated patients.

b) Carbohydrate Modulation of TSH Binding and Graves' IgG TBI

The effects of glucose, fucose, galactose and N-acetyl-neuraminic acid (NANA) on the binding of ^{125}I-TSH and binding inhibition by normal and Graves' IgG, were studied in particulate human crude thyroid membrane preparations.

The monosaccharides were dissolved in different Tris concentrations in order to achieve a final solution pH of 7.4. Glucose, fucose and galactose were dissolved in 25 mM Tris, pH 7.4 and N-acetyl-neuraminic acid in 0.6 M Tris, pH 11. NANA dissolved in 25 mM Tris, pH 7.4 gave a final solution pH of 3. For control purposes, the first 3 monosaccharides were also dissolved in 0.6 M Tris, pH 7.4 and the neuraminic acid in 0.6 N NaOH. Controls were included in all binding assays and consisted of the appropriate buffer in the absence of the monosaccharide.

Neither glucose, fucose nor galactose at concentrations ranging from 20 to 80 mM produced any significant effect on the binding of ^{125}I-TSH to its receptor (Fig. 18). Using the same carbohydrates at the highest concentration dissolved in 0.6 M Tris, pH 7.4 binding of ^{125}I-TSH varied between 80 to 91% of the maximum binding in the absence of the test material.

N-acetyl-neuraminic acid produced a specific concentration dependent, saturable increase in binding of labelled hormone to the receptor, which was maximal at 40 mM NANA (Fig. 19). The effect was parallel, although less marked in solubilized thyroid TSH receptor preparations. Final assay Tris concentrations of 0.05 to 0.125 M, produced a decrease of the ^{125}I-TSH binding (Fig. 20). To study the

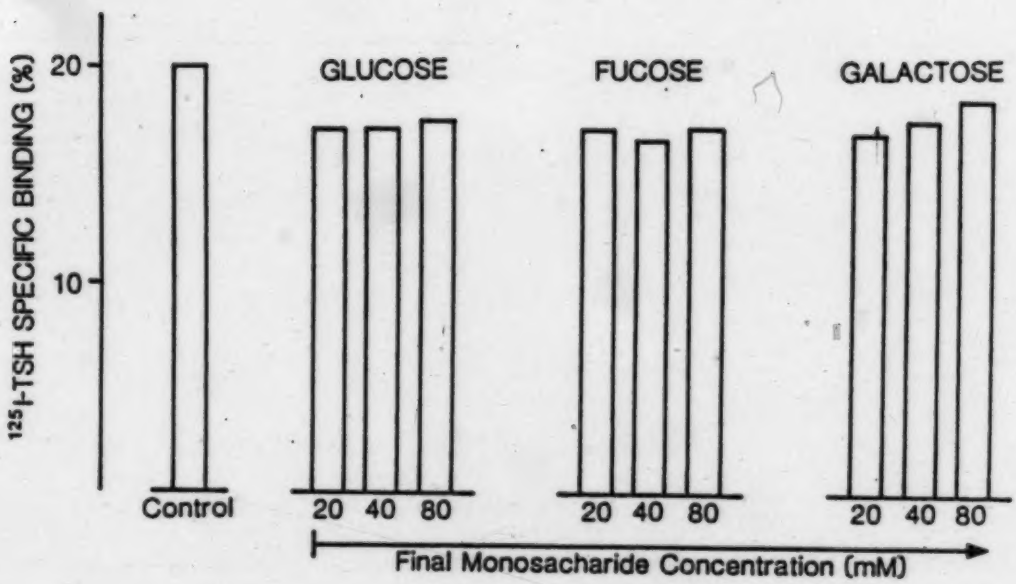


Figure 18. Effects of the presence of glucose, fucose and galactose on ^{125}I-TSH binding to TSH-R (CM). Varying concentrations of glucose, fucose, or galactose produced no significant changes in specific ^{125}I-TSH binding.

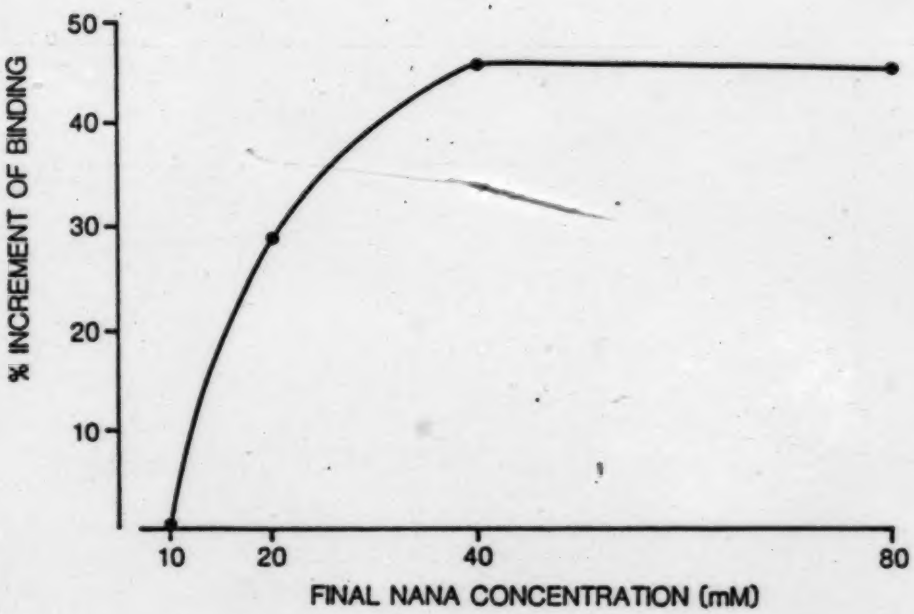


Figure 19. Effect of NANA on ^{125}I-TSH binding to CM. Binding assays were performed in the presence of a constant concentration of Tris (0.075M).

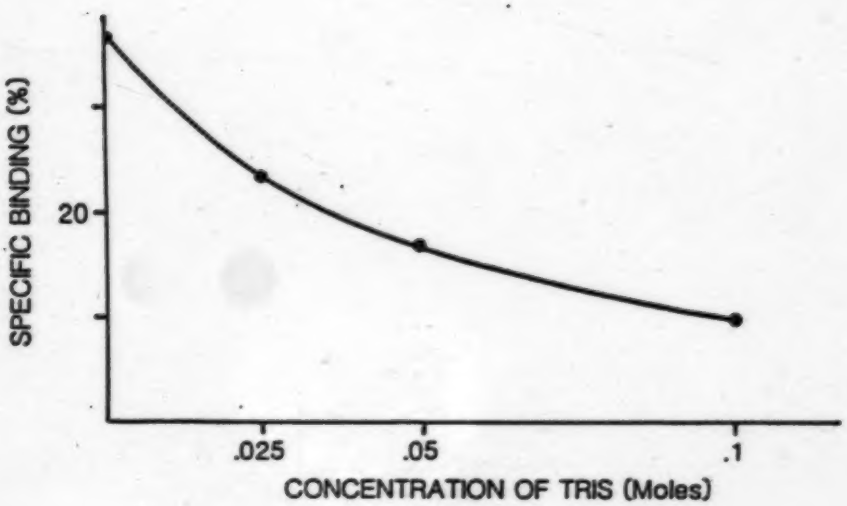


Figure 20. Effects of different concentrations of Tris on ^{125}I-TSH binding to CM.

possibility that the changes observed in the presence of NANA could be attributed to a non-specific effect counteracting the Tris inhibition, NANA was dissolved in 0.6 N NaOH and tested in the particulate thyroid preparation. The presence of NANA again produced an increase in specific binding of ^{125}I-TSH by 32% at 40 mM final assay carbohydrate concentration. As a control, citric acid was dissolved in the same Tris solution to a final pH of 7.4 to test its effects on the binding assay. The presence of citric acid did not produce the increment in binding observed with NANA. These results are summarized in Table 5.

Purified IgGs from 5 normal controls and 6 Graves' patients were tested for their ability to inhibit ^{125}I-TSH binding under standard conditions, in the presence of a final assay Tris concentration of 0.075 M and 40 mM NANA. Under standard conditions, Graves' IgG had a TBI effect of 47.2 $\pm$ 11.5, compared to normal IgG TBI of 24.2 $\pm$ 6.5. In the presence of Tris alone, both groups of IgGs had a more marked effect on inhibiting the ^{125}I-TSH binding, which was further enhanced by the presence of NANA (Fig. 21). The modulations produced by the presence of the monosaccharide were parallel in both the normal controls and in the Graves' IgG group.

c) Graves' IgG TBI in TSH Receptor Preparations from Different Animal Species

Zakarija had previously described 2 alternate actions of IgG from various patients with Graves' disease, with respect to the TBI effect on human thyroid TSH-R (Zakarija 1983). She concluded that the results could probably be attributed to the presence of 2 different

Table 5

Effects of Carbohydrate and citric acid on ^{125}I-TSH binding to particulate human thyroid membrane preparations, under various conditions.

	Concentration (mM)	Percent of the Maximal Specific Binding of ^{125}I-TSH In 0.075 M Tris	In 0.025 M Tris
Glucose	80	80	89
	20		85
Fucose	80	91	86
	20		85
Galactose	80	82	93
	20		84
NANA	40	183	145*
Citric Acid	80	84	

* in the presence of NaOH to give a final pH of 7.4.

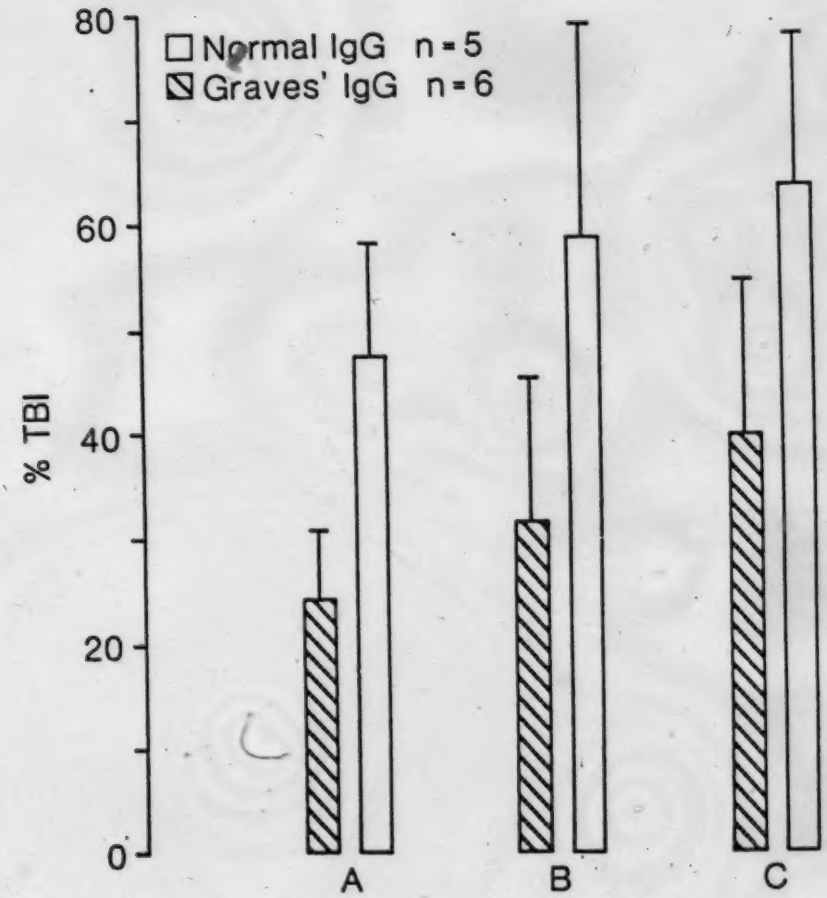


Figure 21. TBI effects of normal and Graves' IgG. A: under standard conditions, B: in the presence of 0.075 M Tris and C: in the presence of 40 mM NANA in 0.075 M Tris. Bars show mean % TBI and one standard deviation.

immunoglobulin G's, of which one augmented ^{125}I-TSH binding to the human receptor, whereas the other inhibited it. The effects were observed at different IgG concentrations; the first one demonstrated an enhancement of TSH binding at concentrations from 10 - 100 µg/ml, whereas the TBI effect was noticeable when the total IgG concentrations were within the 200-1000 µg/ml range. The first effect of ^{125}I-TSH binding enhancement could be seen only with particulate preparations and was not detectable employing solubilized TSH-R. A similar biphasic effect of Graves' IgG was earlier found by Edmonds et al. (1970), although they found an increase in thyroid cell stimulation at low IgG concentrations that was abolished at higher IgG concentrations in the medium.

This bipolar action of an IgG described by Zakarija was studied further. The TBI and enhancement activities were tested in TSH-R particulate preparations of bovine, porcine (both two month and two year old) thyroids and on guinea pig fat cell membranes since, as the human fat cell membranes have been shown to specifically bind ^{125}I-TSH (Teng et al. 1975, Mullin et al. 1976b, Kishihara et al. 1979). These have also been used for partial purification of Graves' IgG (Endo et al. 1981) and have been shown to stimulate the mitogenic response of Graves' lymphocytes specifically (Eguchi et al. 1983).

Particulate TSH-R preparations were made from other animal sources by the same method as used for human thyroid. Protein yields were similar to those obtained for human thyroid and similar amounts of protein were used in the binding studies. All preparations used showed specific, saturable binding to ^{125}I-TSH although the amount of binding differed.

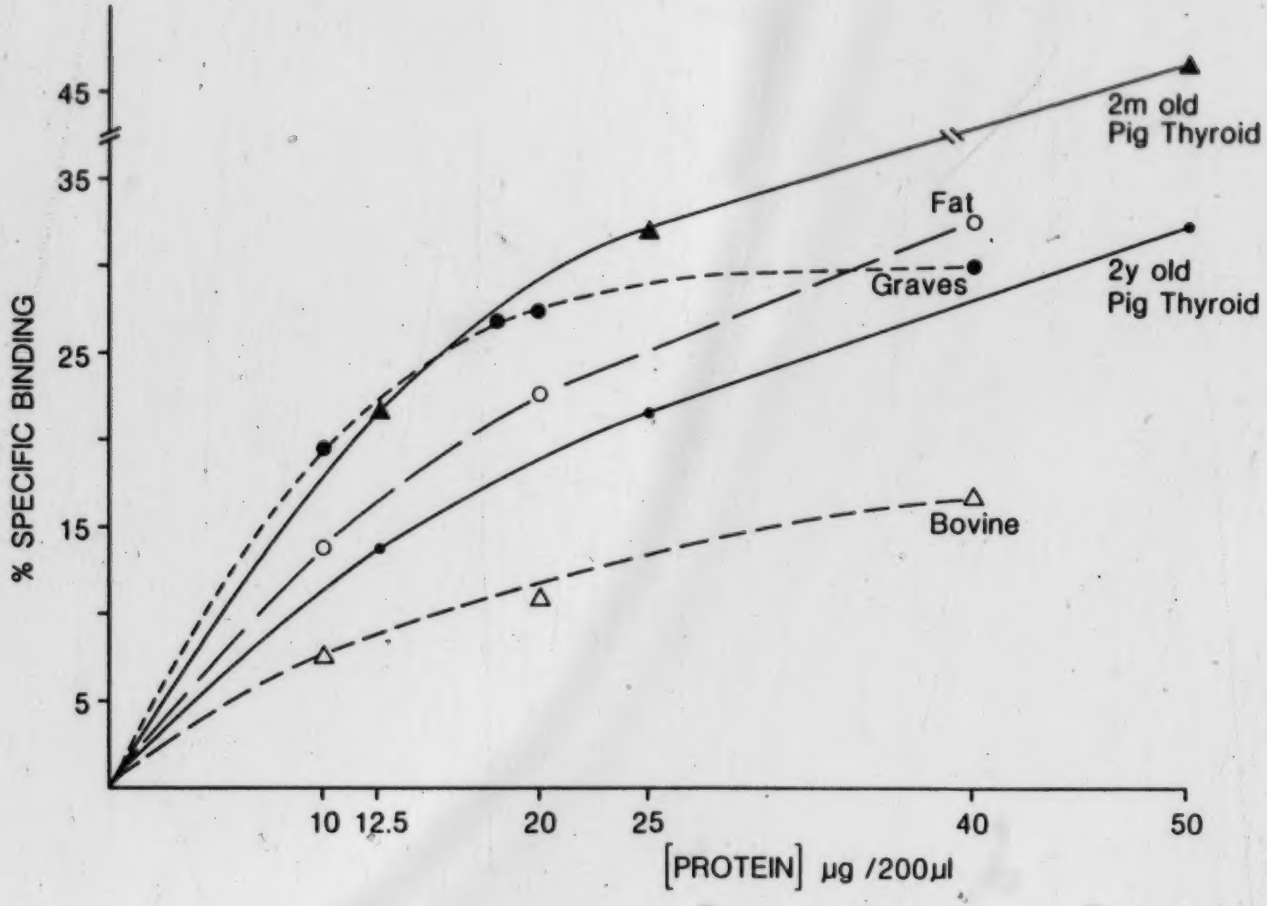
In Fig. 22, the specific binding of ^{125}I-TSH to different protein concentrations of the above mentioned TSH-R preparations is compared. Bovine thyroid membrane preparation gave the lowest binding per µg of protein, whereas the highest binding was found in young (two month old) porcine thyroid.

An IgG from a patient with Graves' disease which did not have the biphasic effect and IgG's pooled from normal human serum were also tested as a control group. The effects of whole IgG, Fab and $F(ab^1)_2$ in equimolar concentrations were studied in parallel. The results obtained from human thyroid TSH-R particulate preparation can be seen in Fig. 23. The biphasic effect was observed in human and to a much lesser extent in bovine thyroid (Fig. 24), but was not present in guinea pig fat cell membranes (Fig. 25), although the TBI effect was comparable to the TBI effect found with human tissue. In the thyroid obtained from the older pig (Fig. 26), a biphasic effect was apparent although not significant, whereas it was completely absent in the two month old pig thyroid preparation (Fig. 27). The inhibitor effect was observed in all tissues tested and the relative potencies of the different IgG fragments were equimolar. The Graves' IgG tested in the control group exhibited similar TBI effects in all tissues examined (not shown).

3. Discussion

Although it is generally accepted that the cause of thyroid hyperfunction in patients with Graves' disease is the binding of an IgG to the thyroid cell, many questions remain unanswered. The fact that the

Figure 22. Comparison of ^{125}I-TSH specific binding to particulate TSH-R preparations made from thyroid tissue of different species and from guinea pig fat cell membranes. Assay conditions and labelled ligand were identical in each case and the same as indicated previously (see Methods).


45
35
25
15
5
% SPECIFIC BINDING
2m old
Pig Thyroid
Fat
Graves
2y old
Pig Thyroid
Bovine
10
12.5
20
25
40
50
[PROTEIN] µg /200µl

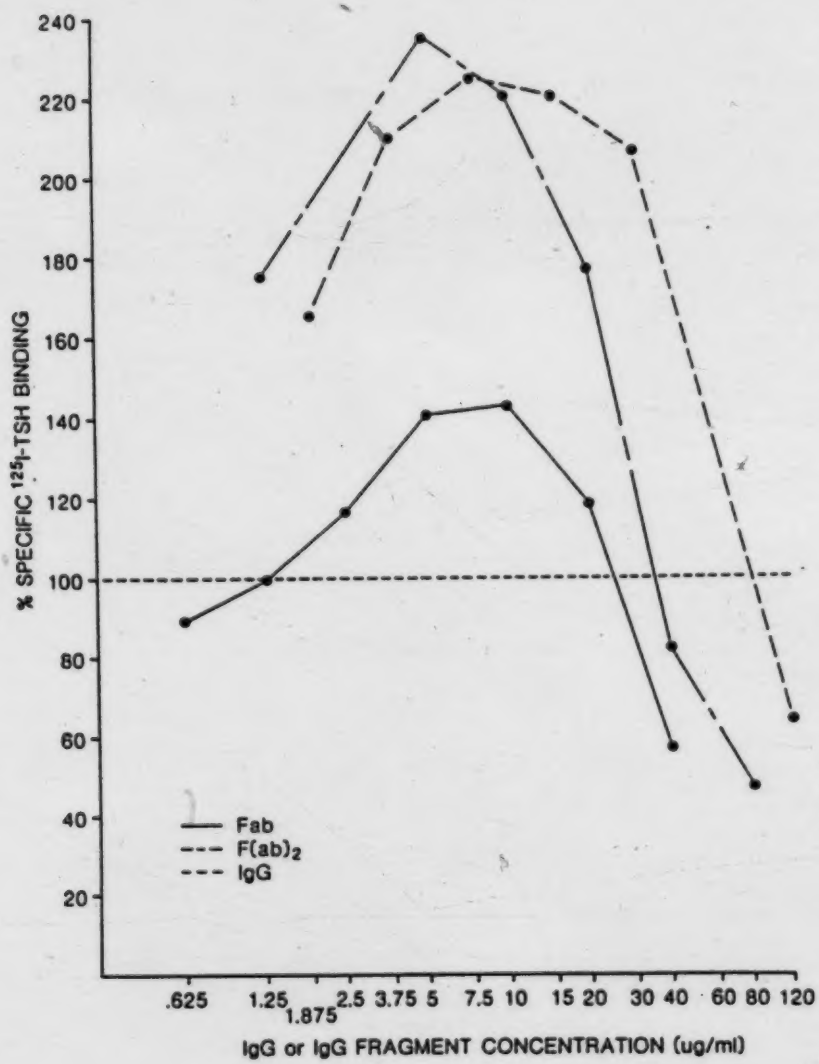


Figure 23. Effects of increasing concentrations of Graves' IgG on human particulate thyroid membrane preparations. Whole IgG, Fab and $F(ab')_2$ fragments from the same IgG were tested in equimoler concentrations.

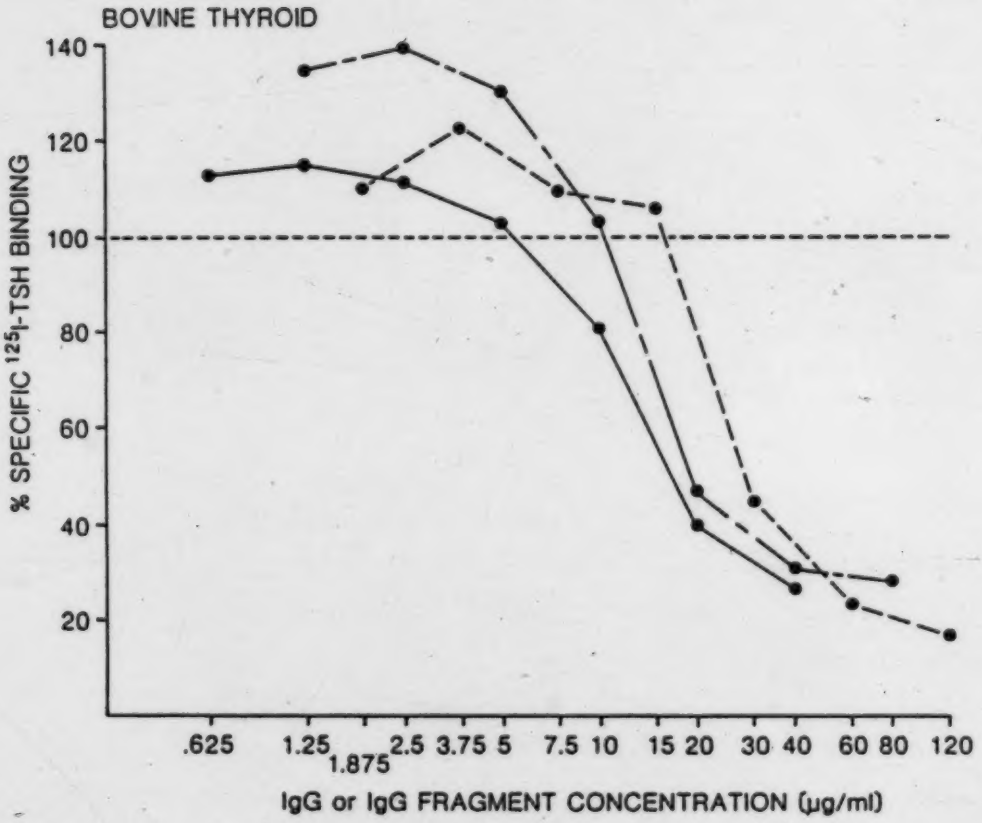


Figure 24. TBI effects of the same IgG tested in bovine thyroid TSH-R particulate preparations. The ^{125}I-TSH binding enhancement effect of IgG at low concentrations is much lower than observed in human thyroid tissue. Fab ——; $F(ab')_2$ — - —; IgG – – – – – – –.

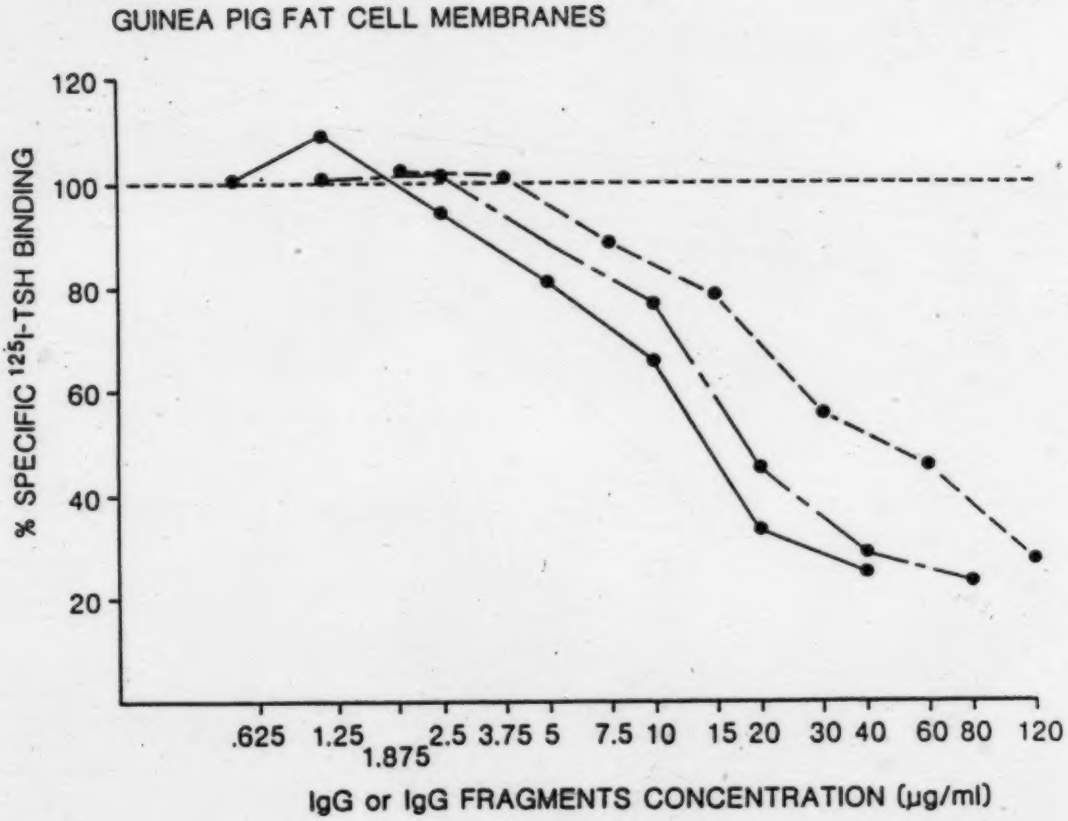


Figure 25. TBI effect of Graves' IgG in guinea pig fat cell membrane preparations. IgG or its Fab and $F(ab')_2$ fragments, tested at the same concentrations as in human thyroid membrane preparation.
Fab ——; $F(ab')_2$ —— - ——; IgG – – – – –.

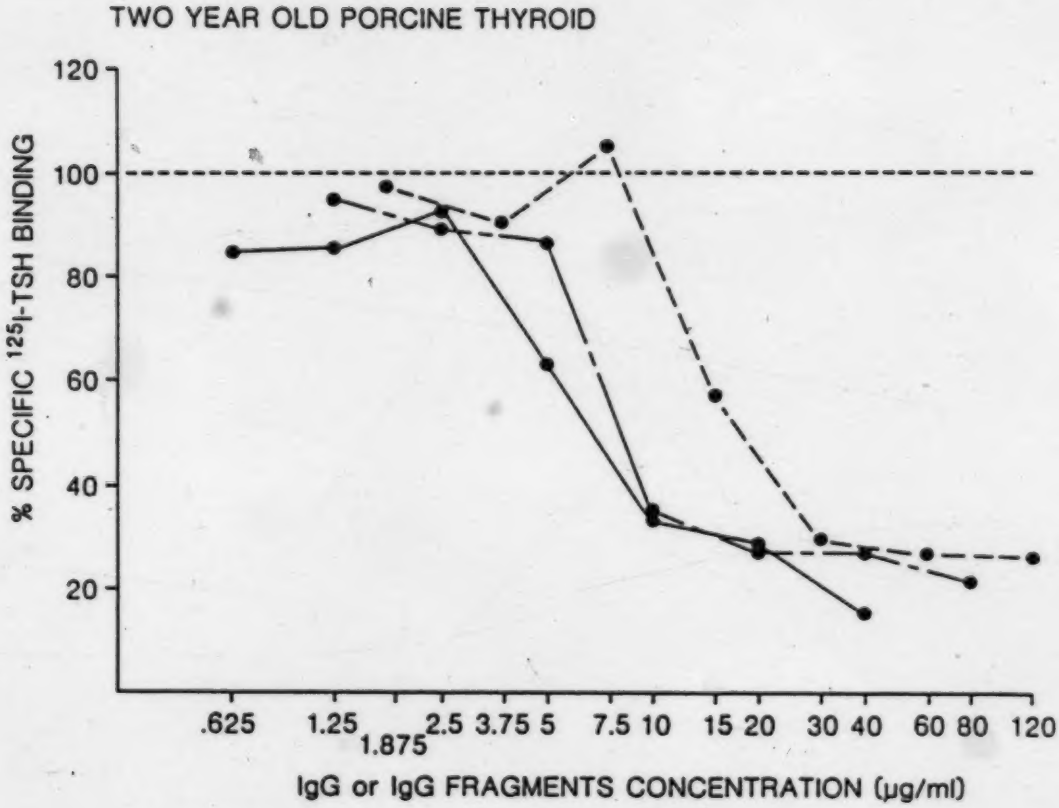


Figure 26. TBI effect of Graves' IgG on 2 year old porcine thyroid membrane preparation. Fab ——; $F(ab')_2$ —— - ——; IgG – – – – –.

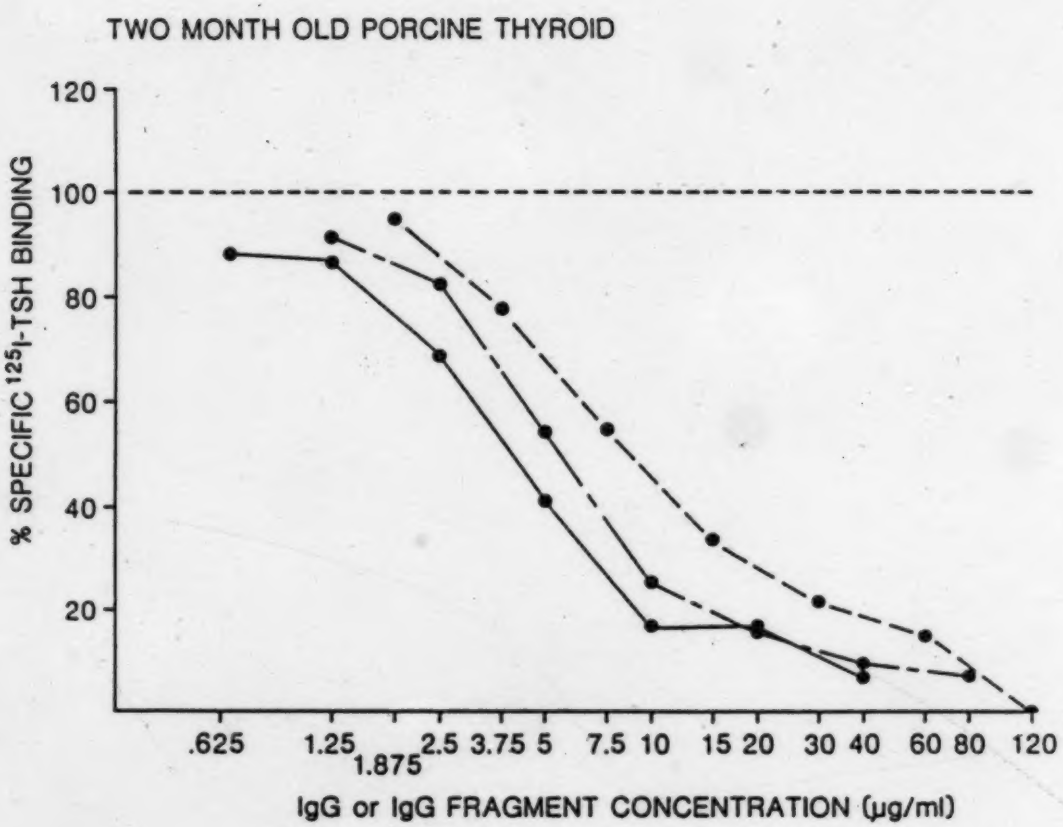


Figure 27. TBI effect of Graves' IgG on 2 month old porcine thyroid membrane preparation. Fab ——; $F(ab')_2$ —— - ——; IgG – – – – –.

TBI effect of Graves' IgG is not universally found and that large discrepancies exist in the findings among authors, even when correlated with the cAMP production assay, suggests the possibility of a broader scope for the interaction of Graves' IgG on the thyroid cell than at the TSH-R binding site for TSH alone.

The first study, in which the TBI of Graves' IgG was tested in two different membrane preparations made from the same human thyroid, suggested that IgGs can bind to different sites, one of which might be to the same TSH-R or to the same binding site as TSH. No advantage in terms of sensitivity was found as a consequence of utilizing different membrane preparations. Since CM contains a higher concentration of intracellular organelles it could be conceivable that they provide non-physiological binding sites for TSH and Graves' IgG. However, in a detailed study by Mehdi et al. (1977) no binding of ^{125}I-TSH was found among the different intracellular fractions. The results could not be explained on the basis of differences among the reagents used, since all assays were done using the same batches of reagents and in parallel, or on the basis of intra and inter-assay variations (coefficient of variation were 6.9% and 9% for CM and PM respectively) and there was a good correlation of TBI values for a given IgG obtained at different times, ($P<0.005$).

Although some authors (Davies et al. 1980, McGregor et al. 1980) have found that the TBI activity of an IgG combined with the HLA type of the patient could accurately predict the outcome of the disease, these results would not support such optimism and would caution against the TBI assay as providing a good index for the clinical course of Graves' disease. The lack of correlation of TBI values

between the two TM was the same regardless of the subgroups of patients analyzed (depending on therapy received or their clinical status).

Gossage et al. (1981), from their study of the TBI effect of Graves' IgG on the patients' own tissue, suggested that the TBI assay did not reliably reflect their pathological effect in vivo. Still, the TBI assay could be useful in some experimental studies, mainly to analyze the site and nature of the interaction between TSH and Graves' IgG with the TSH-R. On these grounds, the analysis of the effects of different pure monosaccharides showed that, although the binding of ^{125}I-TSH to particulate and solubilized receptors was enhanced in the presence of NANA, the TBI effect of Graves' IgG was not specifically affected by this monosaccharide since the minimal enhancement observed was also found among the normal IgGs. The results suggest that TSH and Graves' IgG bind to different sites or that the mechanism of binding is different. The exact mechanism by which NANA produced the enhancement of the ^{125}I-TSH binding is unknown, but as Moore et al. (1976) found, it is possible that the carbohydrate is incorporated into the glycoprotein or ganglioside molecules providing a stoichiometrical change of the TSH-R. The results obtained in this study, with respect to the TSH binding modulation of NANA are in disagreement with the studies of Amir et al. (1973) and Trokoudes et al. (1981). In their reports however, there is no reference as to the buffers used to dissolve the carbohydrates, nor to the final pH of the samples. It is important to emphasize that if NANA is dissolved in 25 mM Tris, pH 7.4, the final pH of the solution is 3. In order to achieve a final pH of 7.4 for the concentrations used, it was necessary to dissolve

the NANA in 0.6 M Tris, pH 11 (final Tris assay concentration .075 mM). The different pH obtained could have varied the final assay pH and hence could have affected the results. It is well known that a variable maximal specific binding have been found using buffers of different pH (Moore et al. 1974, Pekonen et al. 1979, Beall et al. 1979).

It would have been of interest to study the effects of other carbohydrates of the neuraminic acid group, but it was not possible due to the large quantities of Tris required to neutralize the monosaccharide pH (N-glycolyl-neuraminic acid) that resulted in a total maximum specific binding lower than 5%, which in view of the intra and inter-assay variation coefficient (6.9%) was unsuitable for such studies. From the studies of the biphasic effect of Graves' IgG, it can be concluded that some patients have more than one circulating IgG that interacts with the thyroid cell membrane. One IgG would produce a ^{125}I-TSH binding inhibition whereas the second IgG would enhance the binding. The end effect depends on the IgGs' concentrations. The binding sites for both IgGs are different, the second requiring the whole thyroid cell membrane structure for its expression and it appears to be species specific, whereas the first IgG does not have species restriction and its effects are not dependent on the antigenic site being bound to the whole membrane structure. Both effects were found to be due to the Fab fragment of the IgGs, as it had been previously found for the thyroid stimulating antibodies (TSAb) effect (Rees Smith, 1976).

CHAPTER VI

CHOLERA TOXIN-MEMBRANE BINDING AND STIMULATING ACTIVITY

1. INTRODUCTION

Cholera toxin (CT) has been proven to stimulate the cAMP production in different tissues including the thyroid (Mullin et al. 1976a, Zakarija et al. 1980). The hypothesis has been raised that CT binds to gangliosides (GM_1) of the cell membrane, which in the thyroid cells would be part of the TSH-R (Kohn 1977). The theory was founded on the finding of TSH binding inhibition by CT in different systems and on the binding of TSH to the two separate TSH-R components (Meldoesi et al. 1977).

Since Graves' IgG have also been implicated as acting through the same mechanism (by binding to the TSH-R), the use of analogs such as CT for further elucidation of Graves' IgG action could be considered. On the other hand, if CT would indeed bind to the ganglioside component of the TSH-R, it could be used for purification or identification of the whole receptor molecule.

The first step was to ensure that the binding site of CT was the TSH-R for which labelling of CT was necessary in order to perform the appropriate competitive binding studies and to study the binding characteristics of the toxin to the cell membranes.

Purified CT was used for all binding studies as well as for the

cAMP stimulation in thyroid cells.

2. RESULTS

Purified cholera toxin (CT) was initially iodinated by the lactoperoxidase method as in the TSH iodination. Separation of bound and free ^{125}I demonstrated that less than 6% of the radioactivity was in the organic peak. Furthermore, when the iodinated CT was tested in the binding assay to particulate thyroid membrane preparations, specific binding was in the order of only 2-3%.

Following the method of Cuatrecases (1973a), CT could be iodinated with chloramine T (Fig. 28) without loss of its capacity to bind to thyroid and fat membrane preparations. Precipitable iodinated material (TCA precipitation) constituted 6-12% of the total radioactivity. ^{125}I-CT was dissolved in 0.1 M Na phosphate buffer containing 0.1% BSA, pH 7.4 and diluted in 20 mM Tris for the binding assays.

a) Binding of ^{125}I-CT to Thyroid and Fat Cell Membrane Preparations

^{125}I-CT binding to particulate cell membrane preparations was maximal at 5 min incubation at 37°C. Binding was saturable (Fig. 29 and 30) and could be inhibited by excess non-labelled CT (in 0.9% NaCl, 0.5% BSA) (Fig. 31).

Similarly, ^{125}I-CT was found to bind to solubilized human thyroid and guinea pig fat cell membrane preparations in a concentration dependent manner (Fig. 32, 33, 34) that could be inhibited by cold ligand. Assay conditions were different from the ones used for bind-

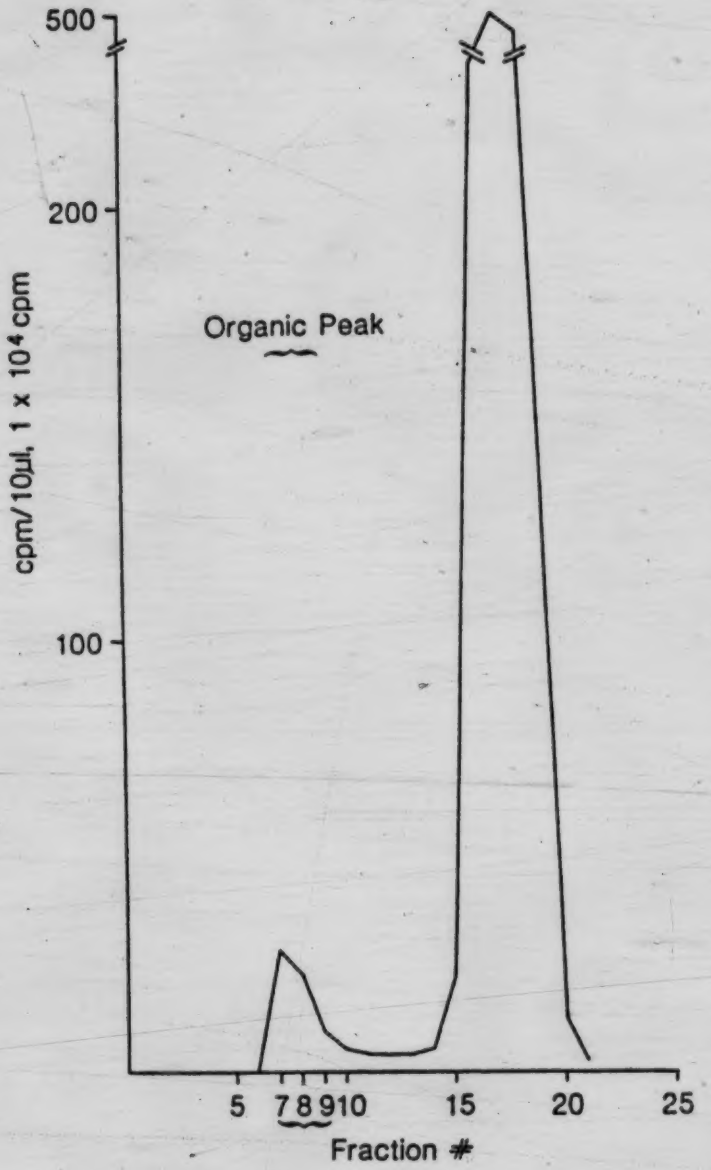


Figure 28. Sephadex G-50 elution profile of ^{125}I-Cholera Toxin (CT). Labelled toxin was eluted at 24°C with 0.1 M sodium phosphate buffer containing 0.1% BSA, pH 7.4. Organic peak fractions were pooled prior to use.

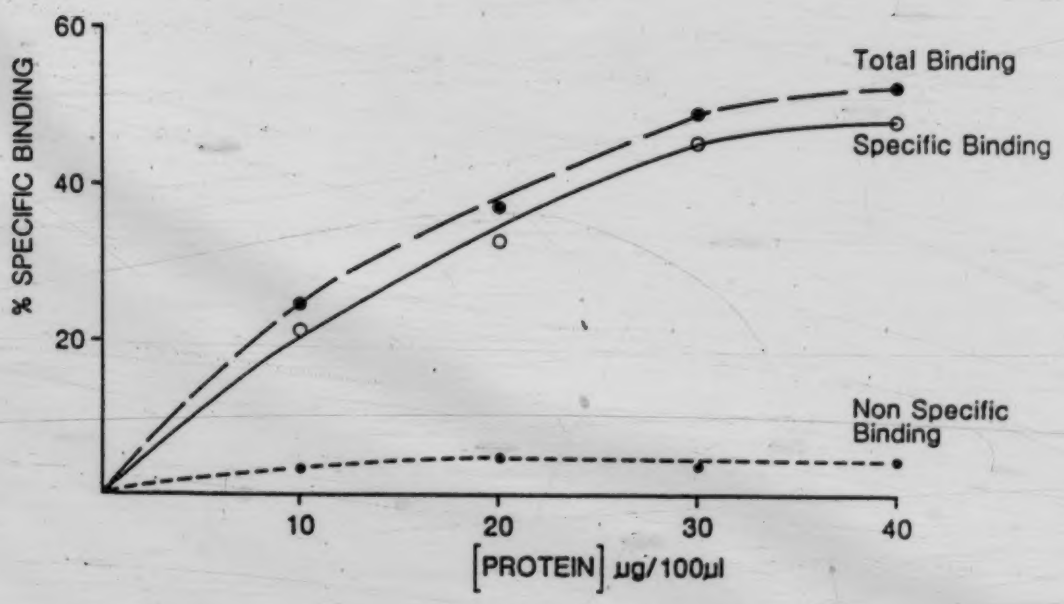


Figure 29. ^{125}I-CT binding to particulate human thyroid membrane preparations. TSH-R preparations were incubated with ^{125}I-CT for 5 min at 37^{o}C, in 20 mM Tris containing 0.5% BSA, pH 7.4. Binding was protein concentration dependent and saturable.

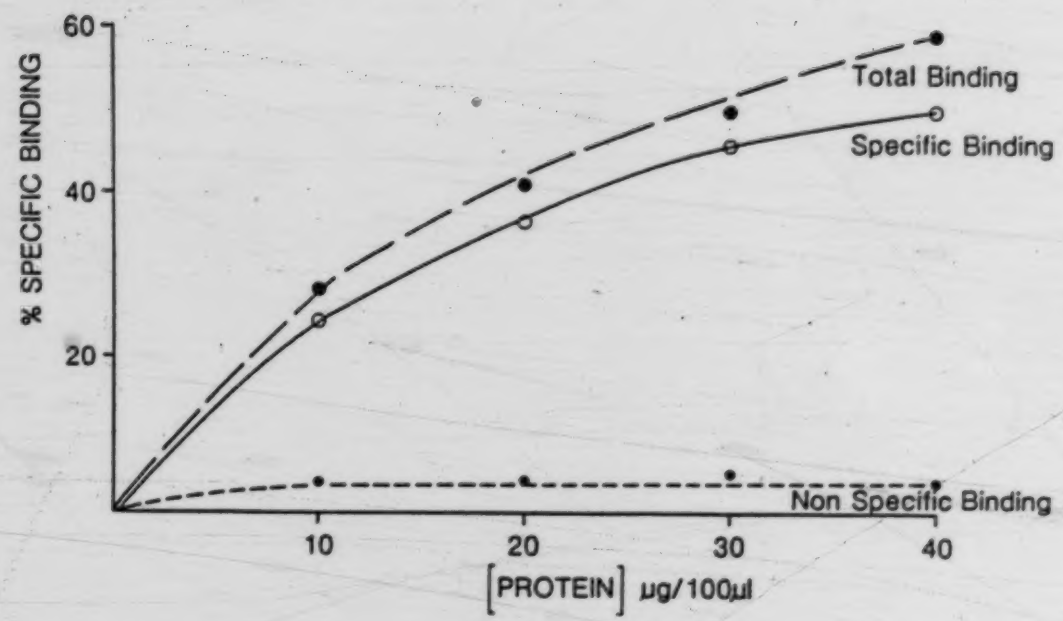


Figure 30. ^{125}I-CT binding to particulate guinea pig fat cell membrane preparation.

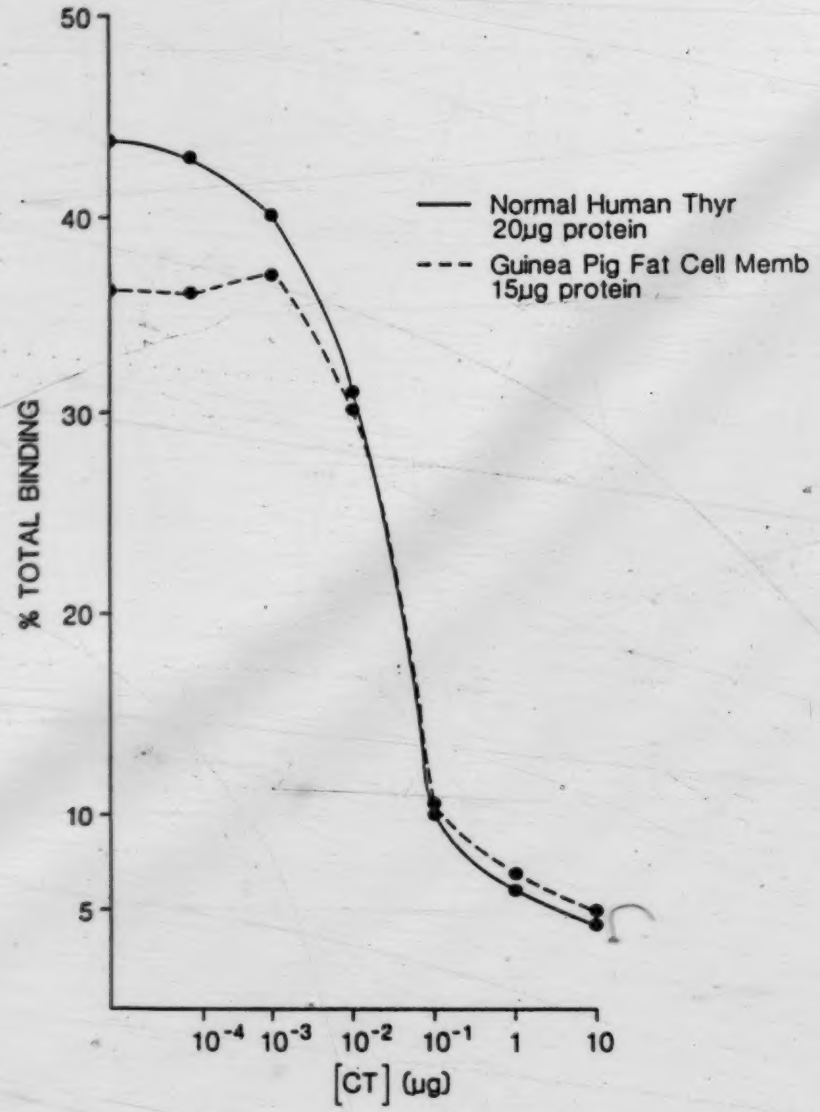


Figure 31. ^{125}I-CT Binding Inhibition by Excess Non-labelled CT.

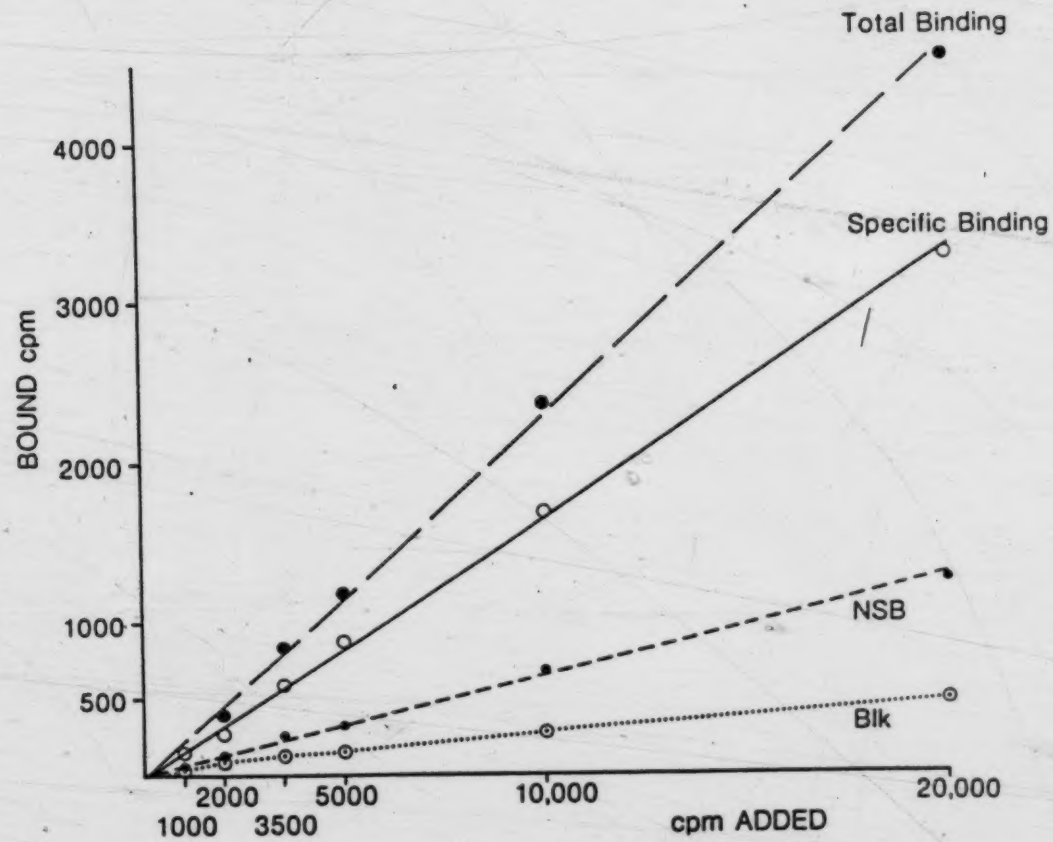


Figure 32. ^{125}I-CT binding to solubilized human thyroid TSH-R. Binding assays were carried out at 37°C for 15 min. Bound ligand was precipitated with 15% PEG 4000 in presence of 0.5 M NaCl without carrier IgG. Under these conditions the NSB was reduced under 10% of the total radioactivity. Addition of carrier IgG to co-precipitate the bound hormone resulted in a NSB higher than 50%. NSB = non-specific binding; Blk = Blank (binding in absence of TSH-R preparation).

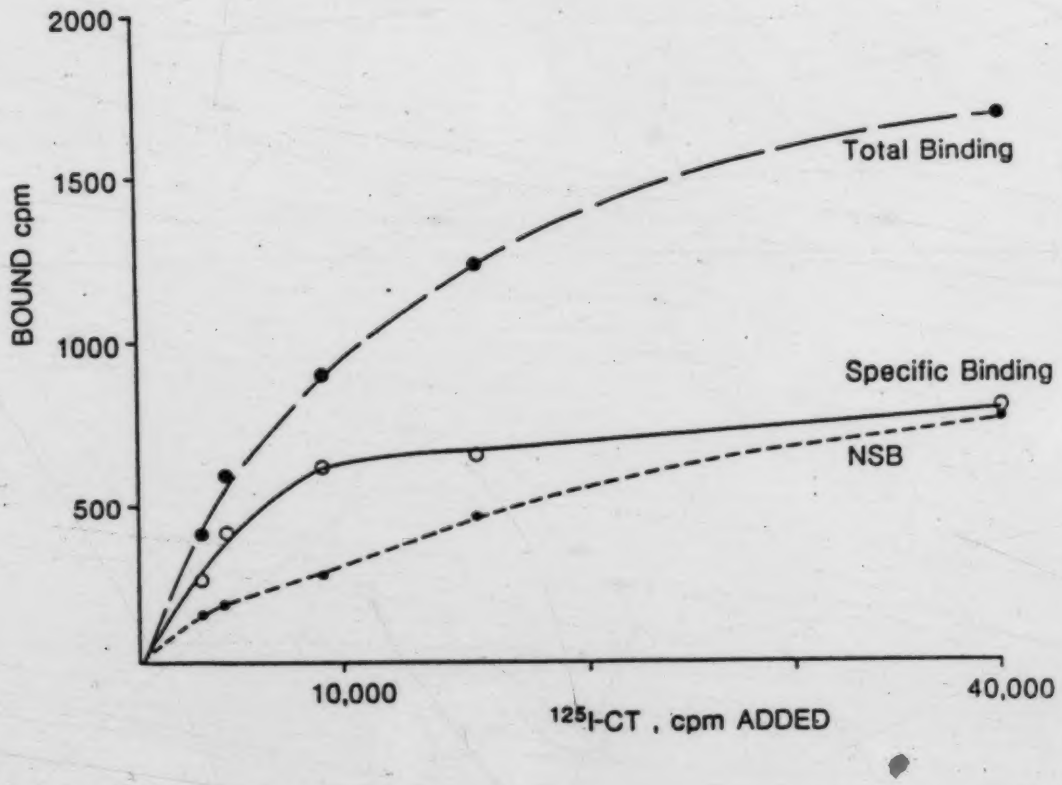


Figure 33. Saturability of ^{125}I-CT binding to solubilized human thryoid TSH-R.

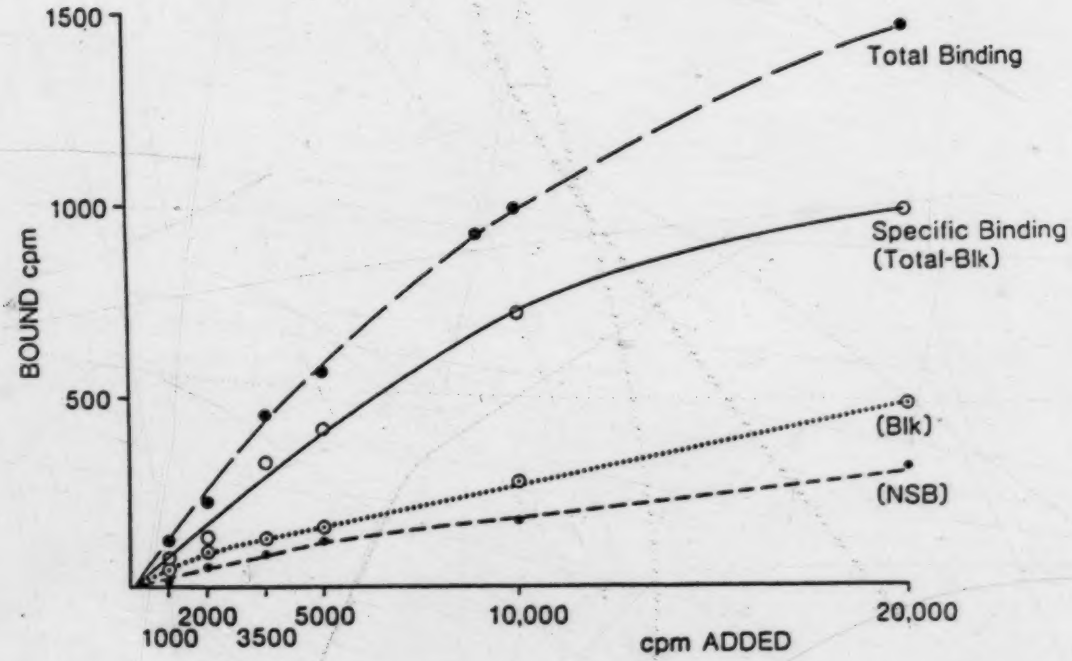


Figure 34. ^{125}I-CT binding to solubilized guinea pig fat cell TSH-R.

ing of ^{125}I-TSH. Non-specific binding (NSB) of ^{125}I-CT, using the later conditions of precipitation, was as high as 50%. To reduce the NSB, a number of experiments were performed and it was found that the omission of normal human IgG to precipitate the complex in the presence of 15% PEG 4000 containing 1 M NaCl (final assay concentrations 7.5% PEG 4000 - 0.5 M NaCl) gave the highest maximum specific binding (MSB) for the lowest NSB. Incubations were carried out at 37°C for 15 min as in the binding of ^{125}I-TSH to the same preparations.

Scatchard plots of the binding data obtained for human thyroid solubilized membrane preparations showed the presence of only one binding site with a Ka of 5.26×10^{9} M^{-1} (Fig. 35).

b) Cholera Toxin Stimulation of c-AMP Production by Thyroid Cells

Since it has been shown that CT can stimulate the production of cAMP in thyroid slices (Zakarija et al. 1980a), the CT preparations used in the binding assay were tested for the capacity to stimulate thyroid cells alone or in combination with TSH. FRTL cells in culture were maximally stimulated at 0.01 µM concentration of CT in the medium producing a 2700% increase in cAMP production with respect to the control. TSH maximal stimulation was obtained with 10 mU giving a 3150% increase in cAMP production. When tested together (0.01 µM CT + 50 µU TSH) no additional effect was observed.

c) Influence of Non-Labelled Ligands ^{125}I-TSH and ^{125}I-CT Binding

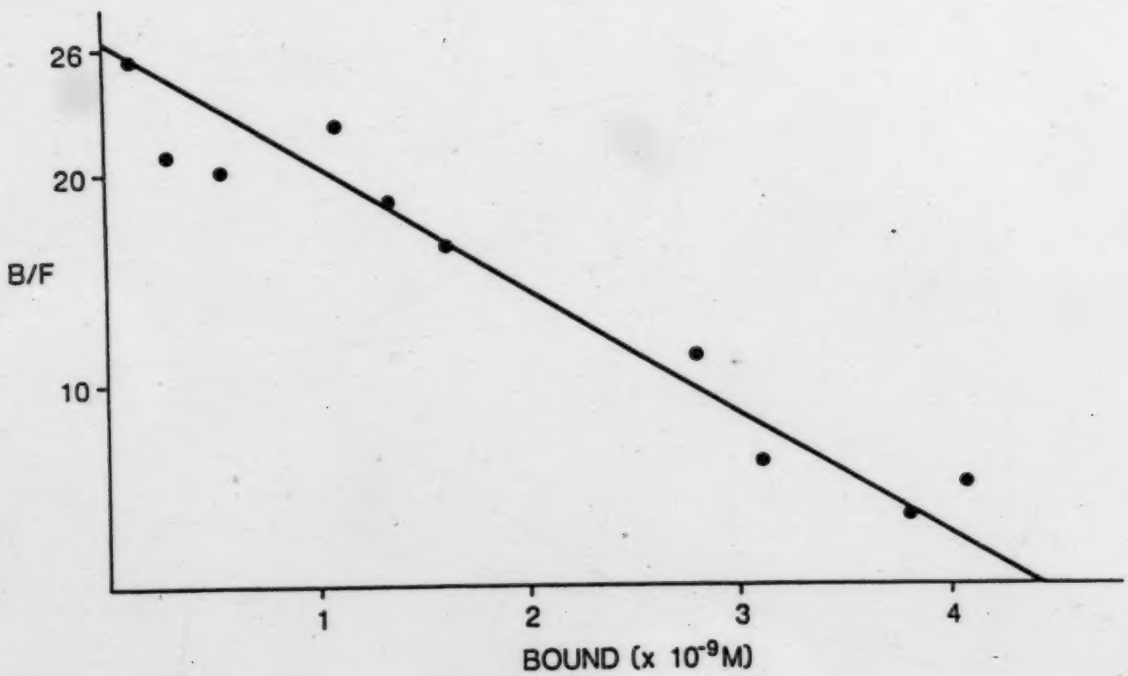


Figure 35. Scatchard plot of ^{125}I-CT binding to particulate human thyroid membrane preparation .

Because of the above observations and previous publications (Mullin et al. 1976a, Zakarija et al. 1980b) suggesting that CT binds to the same TSH receptor, a series of competitive binding assays were done.

The presence of non-labelled TSH did not influence the binding of ^{125}I-CT to thyroid or fat particulate preparations (Fig. 36 and 37). However, when cold CT was added the ^{125}I-TSH binding to particulate thyroid preparations increased to a maximum of 125% (SD $\pm$ 21.1) ($p<0.004$) with a concentration of CT of 10 μM. Since these results were opposite to those found previously (Mullin et al 1976a), and the CT used was dialyzed against saline, (CT comes from the manufacturers in 0.05 M Tris, pH 7.4; 0.001M Na EDTA, 0.003 M NaN_3 and 0.2 M NaCl) the influence of the solution in which CT comes was studied. Non-dialyzed CT produced opposite effects on the ^{125}I-TSH binding to particulate preparations inhibiting the binding to 60% of the maximum specific binding in absence of cold CT. The above solution alone, in the absence of non-labelled CT, produced the same effect (Fig. 38).

The results obtained with dialyzed CT were not dependent on the incubation conditions of the binding assay since the same results were found at 37°C with incubations for 5, 15 and 60 min or at 4°C for 18 h.

Binding of ^{125}I-TSH to solubilized thyroid TSH receptor preparations decreased by 15% in the presence of non-labelled CT (in saline). The effect was not CT concentration dependent (data not shown).

The effects of CT were also studied in the presence of a human IgG, which in low concentrations increases the binding of ^{125}I-TSH to its receptor in the human tissue, whereas the effect is reversed at

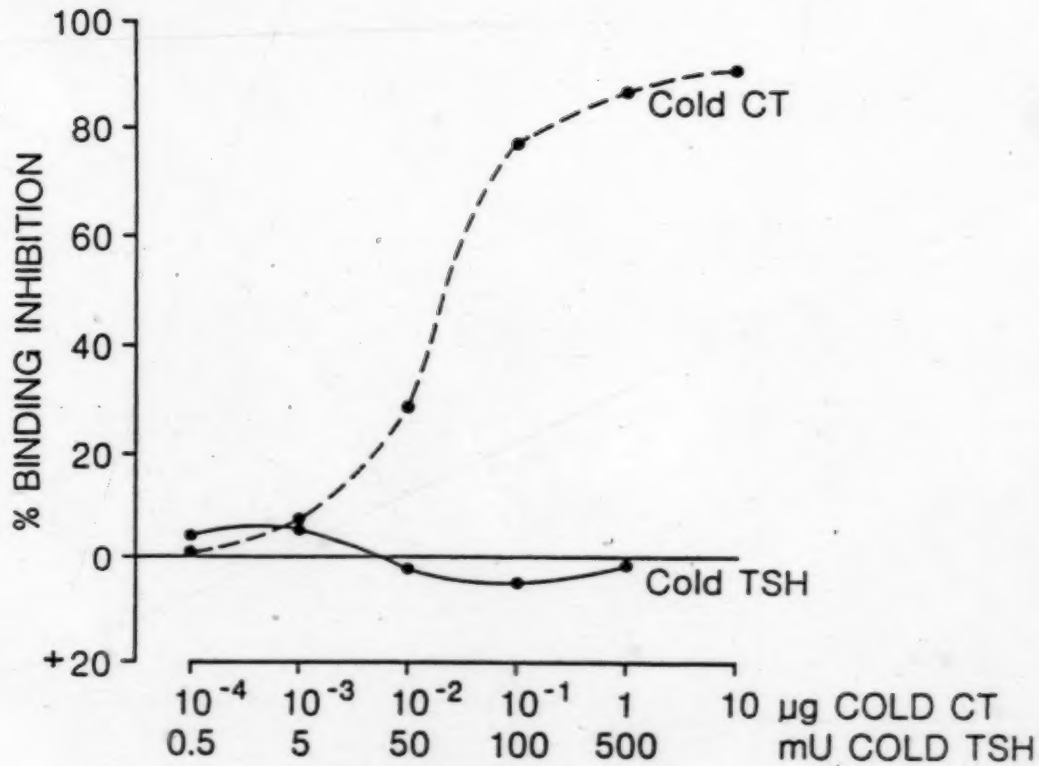


Figure 36. Effects of non-labelled TSH or CT on ^{125}I-CT binding to particulate human thyroid membranes.

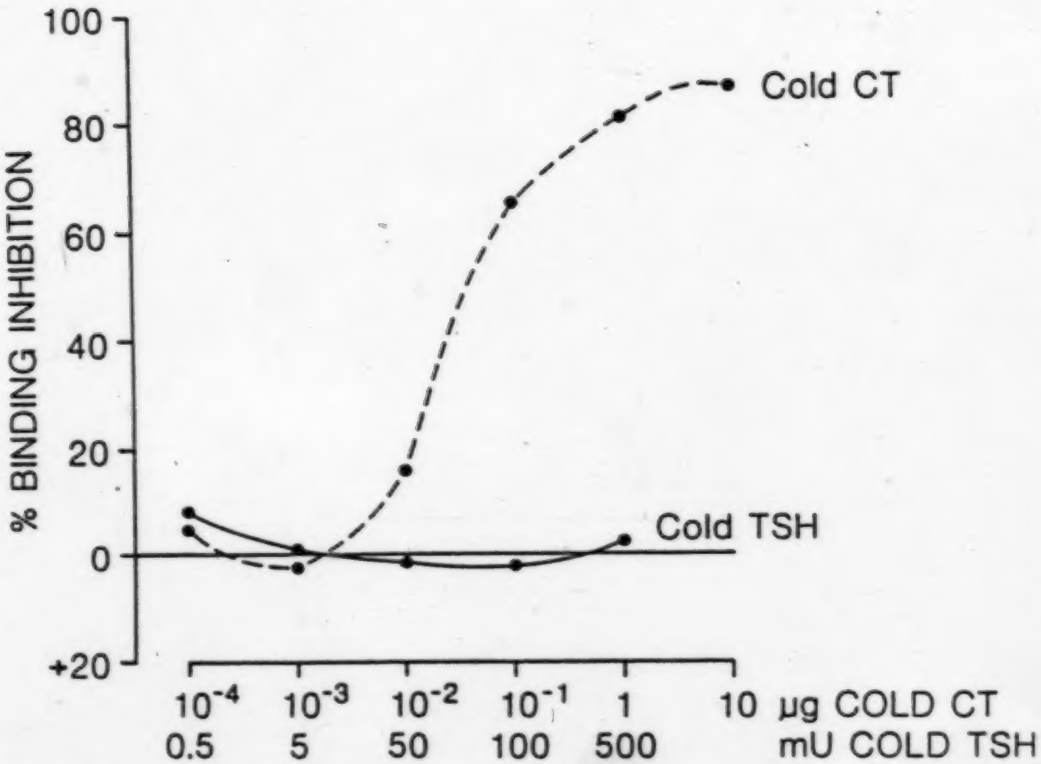


Figure 37. Effects of non-labelled TSH or CT on ^{125}I-CT binding to particulate guinea pig fat cell membranes.

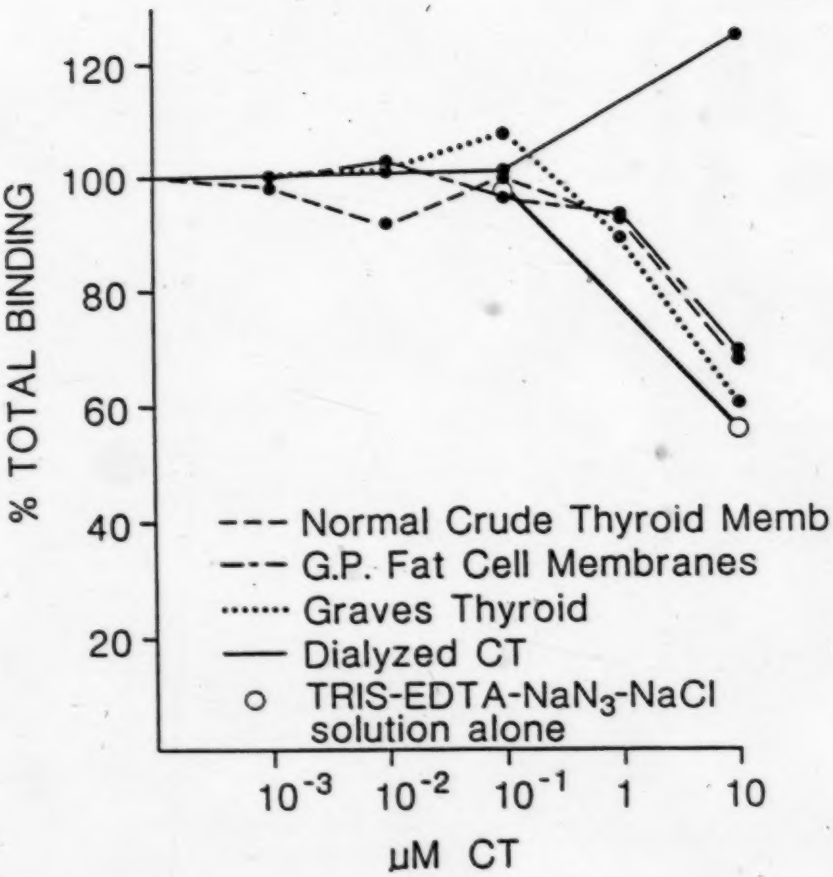


Figure 38. Effects of non-labelled CT on ^{125}I-TSH binding to particulate preparations of TSH-R. The inhibitory effect was due to the buffer in which CT is stored (Tris-EDTH-NaN_3-NaCl) as demonstrated in the binding assay in absence of the toxin. When CT (non-labelled) was dialyzed against saline solution, binding of ^{125}I-TSH was slightly increased. The increment was observed only at the highest cold CT concentrations (10µ M).

higher concentrations. In the former, CT at concentrations from 10^{-3} to 10 µM did not significantly modify the effect of IgG (Fig. 39).

Since CT was able to stimulate cAMP production in the FRTL cells, its effect on binding of ^{125}I-TSH to the cells in culture was also studied. The presence of non-labelled CT at concentrations from 1 to 10 µM did not produce any effect, whereas purified human IgG produced a concentration dependent binding inhibition (Fig. 40).

3. DISCUSSION

Lactoperoxidase iodination is considered a gentle method for protein labelling, whereas chloramine T, usually provides higher percentage of ^{125}I incorporation and hence specific activity at the expense of protein inactivation in some cases.

Because of the relative instability of cholera toxin subunits and their susceptibility to pH and ionic strength changes (Tomasi et al. 1978), lactoperoxidase iodination was attempted first following the method currently used for TSH labelling. Failure to obtain good ^{125}I incorporation to CT and binding of the labelled toxin to thyroid and guinea pig cell membrane preparations prompted the use of the method described by Cuatrecasas (1973a) for CT iodination. The lipolytic activity of ^{125}I-CT had been found to be indistinguishable from the native toxin in experiments performed by the same author.

^{125}I-CT was found to bind specifically to human thyroid and guinea pig fat cell TSH-R preparations in both the particulate and solubilized forms. The binding was comparable to what was previously reported by Cuatrecasas (1973a) in rat fat cells and hepatocytes in

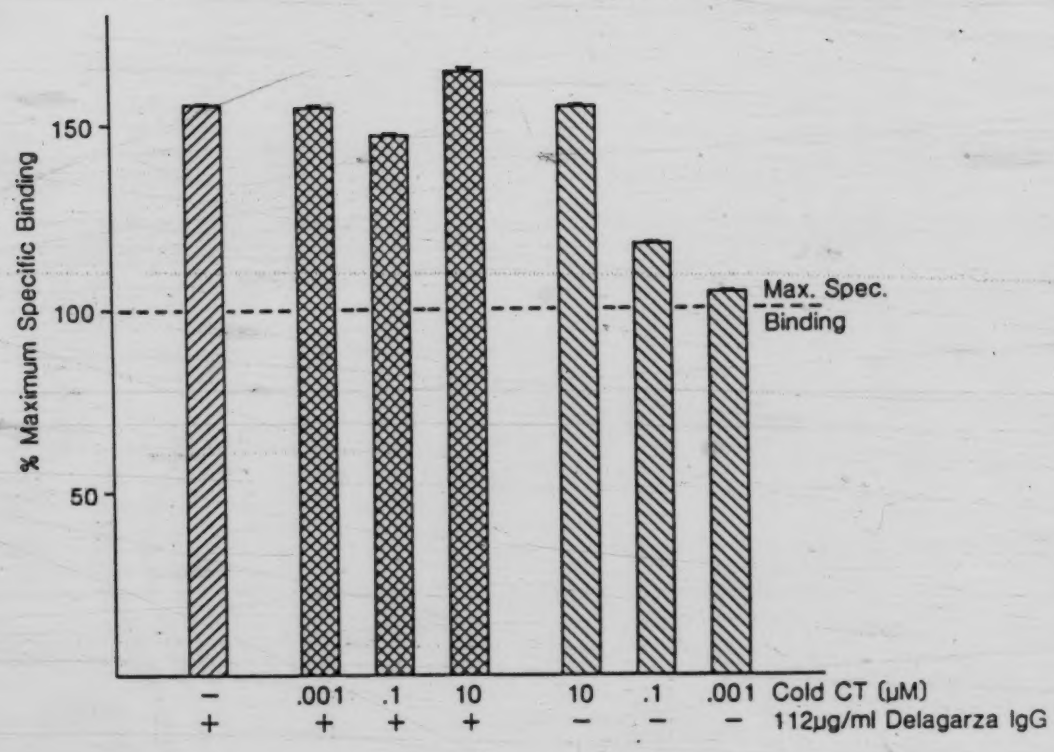


Figure 39. Effects of combined Graves' IgG and CT on ^{125}I-TSH binding to human particulate thyroid membrane preparations. CT was dialyzed prior to its use in the binding assays.

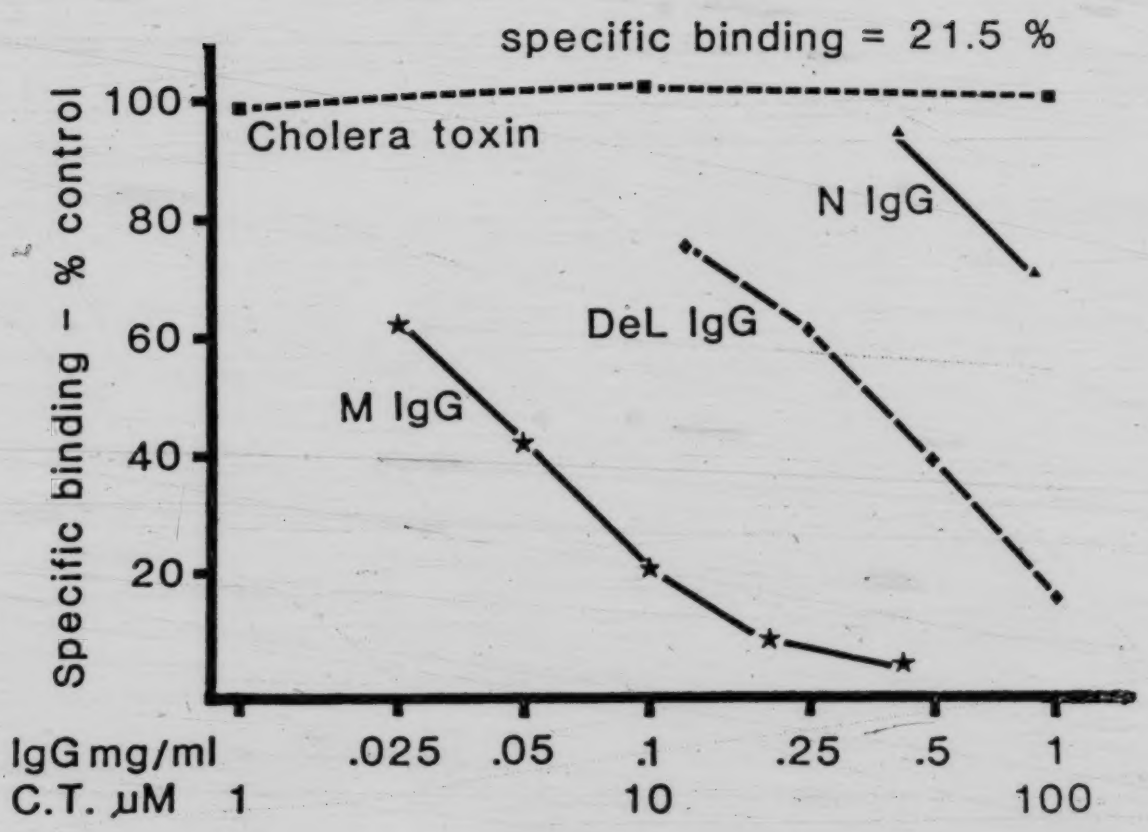


Figure 40. TBI effect of CT on FRTL cells. N IgG = normal human IgG. M IgG and DeL IgG were IgGs from two different patients with Graves' disease.

culture and to intestinal microvillus membranes, by Walker et al. (1974).

Although CT stimulated the production of cAMP in human thyroid slices and FRTL cells as had been described by Mullin et al.(1976)a, Ross et al.(1979) and Zakarija et al.(1980a), presumably by binding to membrane gangliosides, in particular GM (Cuatrecases 1973b, Mullin et al. 1976, Magnani et al. 1980) no significant relationship with the TSH-R was found when studied in the competitive binding assays of ^{125}I-TSH and non-labelled CT.

These results are in disagreement with studies previously published (Mullin et al. 1976a and Zakarija et al. 1980b), in which CT was found to inhibit the binding of ^{125}I-TSH to it receptor, but agree with Pekonen et al. (1980) conclusions that unlabelled CT did not interact with the binding of ^{125}I-TSH when binding assays were performed at 37^{o}C, pH 7.4. Although in initial experiments a 40% binding inhibition was observed in presence of 10 μM CT the effect was due to the buffer present in the CT preparation (50 mM Tris, 1 mM Na EDTA, 3 mM NaN_3 and 200 mM NaCl, pH 7.4) since dialyzed CT did not inhibit, but slightly enhanced ^{125}I-TSH binding and the appropriate dilutions of the above solution produced a 40% ^{125}I-TSH binding inhibition. In the studies mentioned above there was no reference to CT treatment prior to its use in the binding assays, and since it was obtained from the same commercial source, the effects observed were probably due to the presence of the mentioned solution in the binding assays.

The slight increase of ^{125}I-TSH binding to particulate TSH-R only in the presence of high concentrations of CT (10 μM), indicates that the effect is not due to direct interaction of the toxin with the

receptor, since maximal cAMP stimulation was achieved at 1000 times lower concentrations and ^{125}I-CT binding was totally inhibited with 0.06 μM non-labelled toxin. Also the appearance of only one binding site of high affinity for CT and two for TSH on the Scatchard analysis of both binding data to the same TSH-R preparations, favors the existence of different receptors for both ligands. As expected, no interaction between Graves' IgG effect and CT was observed.

Previous reports indicated that GM_1 ganglioside is present in the thyroid cell membranes (Mullin et al. 1976a and Van Dessel et al. 1979) although other gangliosides were found in higher concentrations. Van Dessel and associates in their analysis of gangliosides from bovine thyroid cell membranes were unable to find the most potent ganglioside inhibitor of TSH binding as described by Mullin et al. (1978). The GM_1 ganglioside is the specific receptor for CT (Cuatrecasas et al. 1973b, Taylot et al. 1981) which has been used for the toxin purification and is present in the thyroid cell membrane. Indeed the TSH-R might contain the a ganglioside of CT but this does not imply that TSH binds to the same GM_1.

CHAPTER VII

INDENTIFICATION OF THE THYROTROPIN RECEPTOR AND ITS RELATIONSHIP WITH GRAVES' IgG

1. INTRODUCTION

The localization of specific antigens and their identification in protein complex mixtures constitutes one of the most important approaches to protein isolation and purification. With the availability of pure or purified antibodies, techniques such as Protein A antigen-antibody complex precipitation and binding of antibodies to electrophoretically separated proteins are widely used in different fields. Since Graves' disease is characterized by the presence of one or more abnormal IgG's, and even though other antithyroid IgGs are usually present, the specific antigenic site/s for Graves' IgG could still be identified by such techniques.

Harrison et al. (1979) were able to immunoprecipitate insulin receptors with receptor antibodies from patients with severe insulin resistence. Using the same antibodies, Kasuga et al. (1982) studied the effects of the interaction of insulin with the receptor in cell cultures. Similar studies were done in a cell-free system by Haring et al. (1982) and from these models, they were able to conclude that by binding to the receptor, insulin stimulated the phosphorylation of the 95,000 dalton subunit of its receptor. Protein phosphorylation con-

stitutes the major mechanism by which intracellular events in mammalian tissues are controlled by nervous and hormonal stimuli (Cohen 1980).

Earlier studies by Roques et al. (1975), Giraud et al. (1977) and Field et al. (1977) have shown that thyroid cell membrane proteins can be phosphorylated. There is no proof however, that the TSH-R is a physiological substrate for cAMP-dependent protein kinase which presumably triggers the intracellular events subsequent to its stimulation by TSH (Soderling et al. 1970 and Moore et al. 1974).

Stain detection of electrophorectically separated proteins is poor even with silver stain (Eschenbruch et al. 1982) since silver-stain can only detect 0.1 ng protein/5 mm slot. Because autoradiography of labelled protein is more sensitive when enhancement screens are used, studies were conducted to label rat thyroid cells in culture (FRTL cells) with ^{32}P, which were then subsequently solubilized with Triton N-101 and precipitated with Graves' or normal human and Protein A (Kessler 1976). This technique enables one to establish differences between the antigens precipitated by the two IgGs once separated in SDS-PAGE.

The FRTL cells have previously been shown to be sensitive to TSH stimulation with respect to cAMP production, thyroglobulin formation and iodine concentration (Ambesi-Impiombato 1980).

The finding of a thyroid membrane protein which would be specifically precipitated with Graves' IgG would not be proof that the molecule was the TSH-R. It was not possible however, to identify all proteins that were precipitated by the antibody with respect to their possible relationship with the TSH-R. The only means available at the

present time, to identify the TSH-R is by binding to the labelled hormone. Renart et al. (1979) and Towbin et al. (1979) first described a method for transfer of electrophoretically separated proteins onto different types of paper to render the proteins immobile. This technique had been previously used for DNA studies (Southern 1975) providing an excellent tool for the analysis of cloned DNA.

Once the proteins have been immobilized, they then can be identified by binding to specific antibodies. The Graves' IgGs available are not pure enough to elucidate conclusively the TSH-R localization. In this context, binding of ^{125}I-TSH to electrophoretically transferred proteins was attempted. The technique was developed and the results compared to the binding of Graves' IgG to the same protein map and the proteins precipitated by the different IgGs.

2. Results

a) Phosphorylation of FRTL cells and human IgG protein precipitation

FRTL cells were grown in F-12 modified medium containing 6H for 3 to 7 days prior to conducting the assays, at which time the medium was changed to F-12 + 5H (no TSH) as described in methods. Prior to phosphorylation, the culture medium was replaced by a PO_4-free medium and the cells washed for at least 30 min, followed by phosphorylation with ^{32}P in PO_4-free medium. Cells were phosphorylated with or without variable amounts of TSH for 1 h at 37^{o}C. After stopping the reaction, the cell membranes were lysed with Triton N-101 and the solubi-

lized proteins precipitated with Graves' or normal IgG as described in methods.

SDS-PAGE electrophoretic analysis of the solubilized proteins before IgG precipitation revealed a constant pattern of phosphorylated proteins. Increasing concentrations of TSH did not produce any phosphorylation increment of a specific protein band (Fig. 41) indicating that the process provided incorporation of ^{32}P into the protein molecules was independent of a TSH activated protein kinase action.

When phosphorylated proteins (in the presence or absence of TSH) were precipated with normal human IgG a protein pattern was obtained that differed in several bands from the pattern seen in solubilized non-precipitated proteins (control). The Graves' IgG precipitated the same proteins as well as two additional proteins having molecular weight of 144,000 and 132,000 respectively (Fig. 42). These last two bands were also found in the electrophoretic pattern of supernatants from normal IgG precipitation that had been incubated with Graves' IgG and subsequently precipitated with Protein A.

In some autoradiograms the two bands of 144,000 and 132,000 daltons were faintly discerned in the normal IgG precipitation pattern. Scan densitometry analysis (Fig. 43) revealed that these bands could be significantly enhanced when the proteins had been precipitated with Graves' IgG.

b) Western Blots: Identification of TSH and IgG Binding Sites

Different sources of TSH-R were used to study the binding sites for IgG from patients with Graves' disease and for ^{125}I-TSH.

Figure 41. Electrophoretic profile of phosphorylated FRTL cell membrane proteins. Phosphorylation of FRTL cell membrane proteins was not affected by the presence of increasing concentrations of b-TSH. From left to right, 0, 5, 50, 500 mU/ml of b-TSH in the phosphorylation media. After phosphorylation, cells were lysed with Triton N-101 and the solubilized proteins were submitted to SDS electrophoresis, then followed by autoradiography.

Figure 42. Electrophoretic profile of human IgG - protein A precipitates of phosphorylated FRTL cell membrane proteins. From left to right: 1. - Control, no precipitated proteins, 2. - Normal IgG precipitate, 3. - Graves' IgG precipitate, 4. - Normal IgG supernatant, incubated with Graves' IgG and precipitated with Protein A.

All samples are solubilized proteins phosphorylated in presence of 500 mU/ml of b-TSH.

Two proteins of molecular weight 144,000 and 132,000 (arrows) were precipitated by Graves' IgG more markedly than by normal IgG.

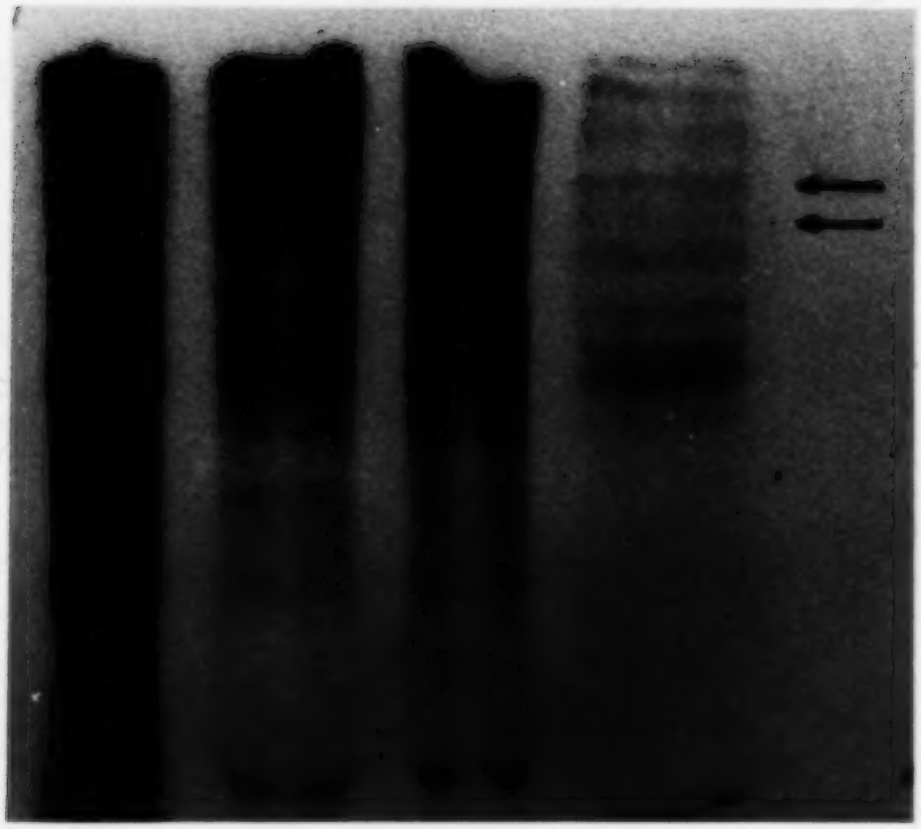
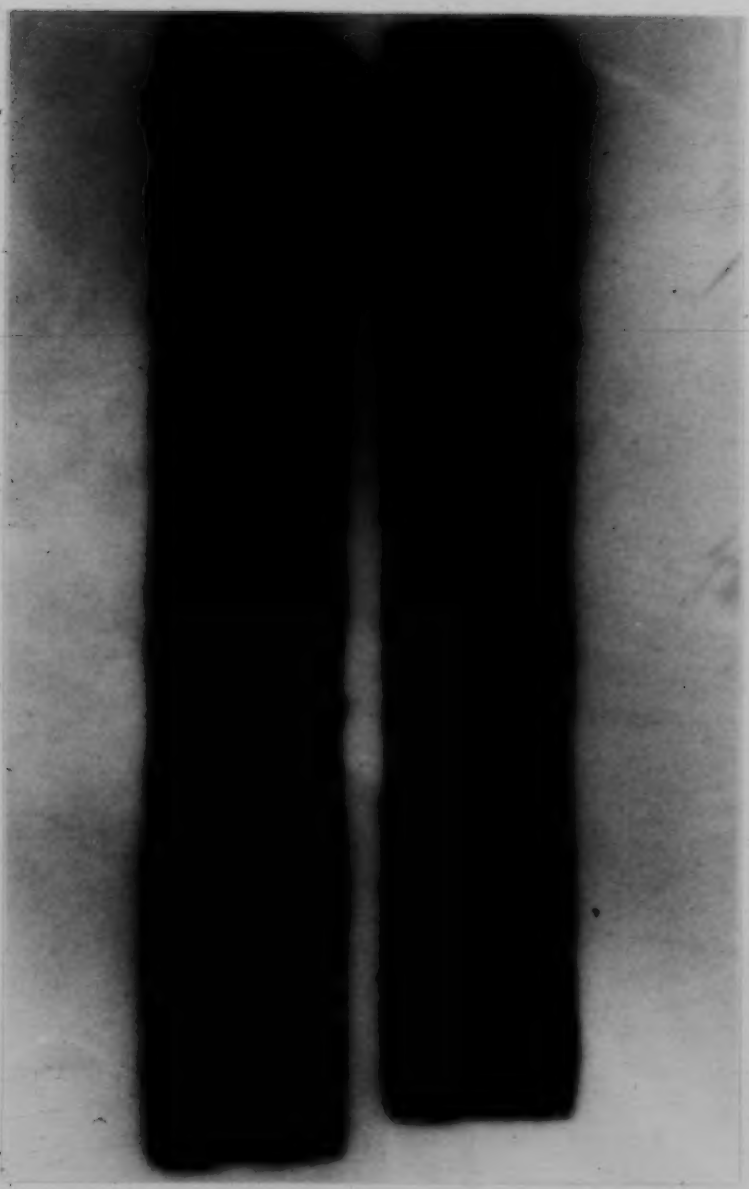


Figure 43. Scanning profile of electrophoresed proteins precipitated by human IgG and protein A. 1. - Normal IgG, 2. - Graves' IgG, 3. - Graves' IgG precipitate of normal IgG supernatant. Arrows indicate the 144,000 and 132,000 molecular weight proteins precipitated by Graves' IgG.

Human thyroid tissue (Triton N-101 solubilized or particulate) was electrophoresed on 10% polyacrylamide-SDS slab gels. Samples were diluted in sample buffer without 2ME. Part of the slab gel containing the standard proteins was stained with either Coomassie Blue or silver stain. The remainder of the gel was placed in the electroblotting chamber immediately following the completion of the electrophoresis for protein transfer onto nitrocellulose paper (NC-paper). Following blotting, the NC paper was cut into strips and each one incubated with different ligands. To avoid background radioactivity, IgGs were diluted in a solution containing 0.25% gelatin and 0.05% Nonidet P40 (see Methods). Bound IgG was detected by adding ^{125}I-goat-anti human IgG and followed by autoradiography. Figure 44 shows the different protein bands of human thyroid tissue that bind Graves' IgG. Numerous bands of different mobilities could be labelled by this method. Since Graves' IgG is not a purified preparation of the IgG (or IgGs) responsible for the disease, it can not be assumed that all proteins labelled are binding sites for the specific Graves' IgG.

To identify the TSH-R, the NC paper strips were incubated with ^{125}I-TSH. No labelled protein bands were found when labelled hormone was incubated under the same conditions and with the same solutions used for IgG binding. Variations of the method included incubation of labelled TSH for 12 h at 4^{o}C under constant gentle rotation and 37^{o}C for 1-2 hours. It was found that incubation of ^{125}I-TSH in Tris - BSA solution, pH 7.4 at 4^{o}C overnight yielded the most consistent results and few bands of radioactivity could be detected by autoradiography. However, the labelled ligand dissolved in 20 mm Tris, 1% BSA bound extensively to the NC paper itself, giving a very dark background.

Figure 44. Graves' IgG binding to human thyroid membrane proteins blotted to NC paper detected by ^{125}I-GAH IgG. Solubilized human thyroid membrane proteins were submitted to SDS-PAGE. Electrophoretically separated proteins were transferred and immobilized in NC paper. Proteins were incubated with Graves' IgG and ^{125}I-goat anti human IgG. Multiple IgG binding proteins could be detected, one of them of 130,000 dalton molecular weight.



The use of 0.05% detergent (Nonidet P40 or Triton N-101) almost completely inhibited the binding of ^{125}I-TSH to the blotted proteins. Several variations were tried and it was found that incubation of the blotted NC paper with boiled non-labelled commercial TSH for 30 minutes at 37^{o}C reduced the background substantially. After incubation with boiled cold hormone, strips of NC paper were cut and placed in separate containers for incubations with ^{125}I-TSH in the presence or absence of cold ligands, IgG, cold LH, hCG, prolactin or cholera toxin.

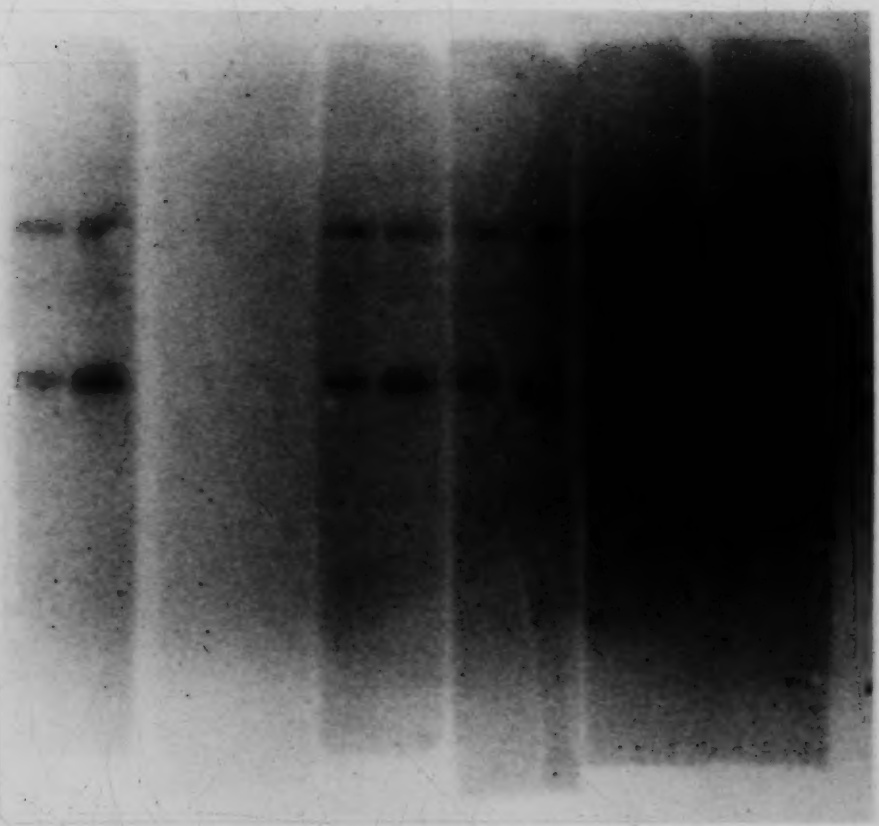
^{125}I-TSH repeatedly bound to two protein bands with molecular weights of 128,000 and 80,000 (Fig. 45). These two bands were consistently detected regardless of the source of human thyroid TSH receptor preparation used (i.e. particulate or Triton N-101 solubilized). In a few experiments an additional band with molecular weight of approximately 95,000 was detected, mainly when particulate thyroid preparation was used as source of TSH-R. Binding of labelled TSH to these protein bands was completely inhibited by the presence of cold commercial bovine/TSH (Thytropar) at concentrations of 0.5 - 1 IU/ml, but not by NIH TSH under the same conditions at lower concentrations. Neither LH, hCG, prolactin, normal IgG, Graves' IgG or cholera toxin were able to inhibit the binding of ^{125}I-TSH to those protein bands (Fig 45). In some experiments samples used in electrophoresis were diluted in sample buffer containing 10% 2ME. When blotted proteins were incubated with ^{125}I-TSH, the same labelled bands were found as in the absence of 2ME in the samples.

Since Erickson et al. (1982) has suggested the possibility of re-using the NC blotted proteins after washing off the ligands, as the

Figure 45. ^{125}I-TSH binding to human thyroid cell membrane proteins blotted to NC paper. NC paper transferred proteins were incubated with ^{125}I-TSH in the presence of (from left to right): 1. - Alone, 2. - Thytropar (b-TSH) 500-1000 mU/ml, 3. - NIH b-TSH 200 mU/ml., 4. - LH.,

5. - HCG., 6. - Prolactin.

^{125}I-TSH specifically bound to 2 protein bands of mol. weights 128,000 and 80,000.



proteins remain stable, experiments were conducted in which the blotted proteins were first exposed to ^{125}I-TSH binding, washed after autoradiograms were obtained, and then exposed to human IgG followed by ^{125}I-goat anti-human IgG. Among the multiple bands labelled by this method, not one corresponded to the bands labelled by ^{125}I-TSH (128,000 and 80,000 daltons).

3. DISCUSSION

Precipitation of labelled proteins had proved to be a valuable method for the identification and isolation of specific proteins (Kozlovskis et al. 1982 and Kasuga et al. 1982). In our system, the Protein-A precipitation of phosphorylated cell membrane proteins from the FRTL cells, that had previously been studied for their capacity to respond to TSH and Graves' IgG, allowed the identification of two different protein bands which bound to Graves' IgG, but not to normal IgG.

Phosphorylation was chosen because of its prominent role in cell function, and more specifically, because other authors have found that receptors are actively phosphorylated as a consequence of their interaction with their specific hormone (Gordon et al. 1977, Cohen et al. 1980, Kasuga et al. 1982). Moreover, a subunit of the insulin receptor has been recently identified as a protein kinase. Although protein kinase activity in thyroid slices is stimulated by TSH (Field et al. 1975 and Giraud et al. 1977) and more directly by cAMP (Yamashita et al. 1972), there is no proof that the TSH-R is phosphorylated as a result of TSH binding. Using a wide range of TSH concentrations which

were active in stimulating the production of cAMP in the same cells (0-500 mU/ml), and which showed specific binding to the TSH-R when ^{125}I-TSH was used, no enhanced phosphorylation of any band was observed. Slight differences might have been found if a lower range of TSH concentration, from 0.005 - 5 mU/ml, had been used since cAMP production increases proportionally up to a certain concentration and then reaches a plateau. As seen in Fig. 41 however, no distinction was visible between the phosphorylated proteins in the presence (5 mU) or in the absence of TSH even in 2 dimensional electrophoresis (data not shown). The findings suggest that either TSH-R is not an endogenous substrate for TSH activated protein kinase or that in order to achieve such enzymatic phosphorylation, other incubation conditions should be used. This is somewhat surprising in view of the fact that some authors (Kasuga et al. 1982) have reported insulin receptor phosphorylation under the same conditions. The results suggests that the phosphorylation obtained is the consequence of passive incorporation pf ^{32}P into phosphate deprived membrane protein.

It is still possible that none of the proteins labelled correspond to the TSH-R or its fractions. This possibility remains open until identification of the TSH-R can be achieved by the other means.

Although we cannot assume that all membrane protein are phosphorylated and, moreover, visible on audioradiography after their electrophoretic separation, the results obtained are interesting in view of the differences found when Graves'IgG were used to precipitate the protein as opposed to normal IgG precipitation. Because of the limitations of the technique, the results do not demonstrate that these two protein bands, precipitated with Graves' IgG are the speci-

fic binding site for IgG, neither can it be assumed that they are the only Graves' IgG antigens.

As demonstrated by binding of IgG to blotted proteins (Fig. 44), there are numerous binding sites among the human thyroid solubilized proteins. Again, this does not imply that all those proteins represent antigens specific for Graves' IgG, nor do they give any further knowledge of the TSH-R and its possible interaction with Graves' IgG. The numerous bands labelled by binding to human IgG and ^{125}I-goat anti-human IgG to blotted human thyroid membrane proteins indicate that precipitation of specific proteins by IgG - Protein A (as used in the FRTL phosphorylated cells study) is less sensitive than direct labelling of blotted proteins. The most important limitation of the precipitation procedure is the large amount of IgG necessary to obtain good antigenic precipitation, which in turn interferes with mobility of proteins on electrophoresis.

It is of interest to compare the binding of Graves' IgG to thyroid proteins by the two methods. In this regard, of the two phosphorylated proteins precipitated by Graves' IgG, the 132,000 dalton protein was also labelled by ^{125}I-goat antihuman IgG bound to Graves' IgG, even though the source of thyroid proteins used was different in the two techniques (FRTL cells in the first case and human thyroid solubilized membrane proteins in the blotting technique).

Of importance is the use of ^{125}I-TSH binding to blotted proteins to identify the TSH-R. As far as the author is aware, this approach has never been used before, although a similar technique was used by Tawata et al. (1982) who were able to identify the calmodulin-binding protein of bovine thyroid plasma membrane by direct binding of label-

led calmodulin to slab gel electrophoresed proteins. In the current experiments two bands were found which specifically bound to labelled hormone. Binding could not be displaced by other hormones such as HCG, LG and prolactin, but was specifically inhibited by the simultaneous incubation with cold commercial TSH. The failure of NIH b-TSH to inhibit binding of labelled TSH could be attributed to the lower concentrations used, in contrast to the concentration of commercially available TSH. Possibly larger concentrations (which were not easily available) would be necessary to obtain a similar degree of binding inhibition. The binding of ^{125}I-TSH was localized on the 128,000 and 80,000 dalton proteins. Since large polypeptides and protein complexes become dissociated during the electrophoretic procedure the two TSH-binding proteins could be part of a TSH-R molecule. Other TSH-R fractions might have been dissociated by the procedure, and if they are not direct binding sites for the hormone would have become unidentifiable by this technique.

In some experiments, the same strips of blotted protein to NC paper were used for both ^{125}I-TSH binding and IgG - antihuman IgG binding. This eliminates the possible electrophoretic pattern differences observed from gel to gel and in protein blotting. In these cases, the closest IgG bound proteins to the ^{125}I-TSH bound proteins had molecular weights of 130,000 and 78,500 daltons respectively, thus indicating that the binding sites of ^{125}I-TSH and Graves' IgG are not identical. This conclusion is supported by the fact that incubation of Graves' IgG did not inhibit the binding of ^{125}I-TSH to bands of 128,000 and 80,000 dalton.

When other sources of thyroid membranes were used for the ^{125}I-

TSH binding to blotted proteins (FRTL cells not phosphorylated or guinea pig fat cell membrane proteins) the hormone binding was localized at different m.w. proteins. It seemed that the binding of ^{125}I-TSH identified two different doublets of m.w. 118,000-108,000 and 77,000-70,000, besides the inconsistently found band of 39,000 daltons. Small changes in the electrophoresis run and calculations of molecular weights can account for the differences of the molecular weights for TSH-R from those sources, but most probably, it suggests that the TSH-R molecules of the FRTL and guinea pig fat cell are not identical to the TSH-R of the human thyroid.

CONCLUSION

The initial aim of the project was to obtain a pure preparation of TSH-R, by biochemical techniques and by raising monoclonal antibodies to the receptor and using them for the final purification. The availability of such a preparation would not only have allowed the analysis of the TSH-R interactions with Graves' IgG, but also to investigate the receptor's role in the etiology of Graves' disease.

Neither by TSH affinity chromatography or by Graves' IgG immunoabsorbents, was it possible to obtain a pure preparation of TSH-R. The partially purified receptor was found to have a sedimentation coefficient between 4.2S and 4.9S, but the complete identification and characterization of the molecule was hampered by the presence of thyroid contaminants. Moreover, the degree of purification, as measured by binding to ^{125}I-TSH, was only 140 times with respect to the non-purified material.

The partially purified TSH-R preparation however, was suitable for production of monoclonal antibodies, since large amounts of TSH-R from solubilized human thyroid membrane preparations could be purified by this method. The main difficulty encountered was in the selection of clones. Several screening assays were used and among them, a modified TBI assay which was specific for identification of IgGs against TSH-R. Unfortunately these assays had poor reproducibility. When the monoclonal antibodies were purified and tested, they did not inhibit binding of ^{125}I-TSH. Although some of the monoclonal antibodies stimulated the production of cAMP in thyroid cells, this did not constitute a proof of the antigenic specificity for the TSH-R.

Since total purification of the TSH-R could not be achieved by those methods, its identification among the thyroid contaminants was attempted by electrophoretic separation of the different molecules, followed by binding of ^{125}I-TSH. The transfer of separated proteins to NC paper provided a stable substrate of immobilized proteins, suitable for the binding of labelled ligand. Two proteins of molecular weights 128,000 and 80,000 daltons, specifically bound ^{125}I-TSH. The results indicate that either two different molecules of the human thyroid membranes, are specific receptors for TSH, or that the receptor is only one molecule, with two binding sites for the ligand, which became separated during the electrophoretic procedure, possibly due to the dissociation of receptor subunits.

Although a pure preparation of TSH-R could not be obtained, its interaction with Graves' IgG was investigated. In the first study, IgGs gave different TBI values, depending on the TSH-R preparation used. The fact that the only variable in the assay was the procedure of membrane preparation, and that results did not correlate, suggested that the method used to measure TBI was influenced by other factors than the actual TBI activity of Graves' IgG.

Binding of ^{125}I-TSH was specifically increased in the presence of NANA. This finding allowed to further investigate the relationship of TSH-R and Graves' IgG. No specific effect for Graves' IgG was found in the presence of the monosaccharide indicating that the binding sites of Graves' IgG and TSH were not identical. Still unresolved was the possibility that Graves' IgG would not bind directly to the TSH-R.

Since some IgGs exert a biphasic effect due to the presence of more than one thyroid membrane antibody, it was possible to prove that

these Graves' IgG can bind to the thyroid cell, through a membrane component different than the TSH-R. This second membrane component was found to be specific for human thyroid tissue.

Further demonstration of a non-TSH-R membrane component, being the binding site for thyroid cell stimulators, was provided by the cholera toxin studies. They also indicated that the CT binding site was not the same as the second Graves' IgG (thyrotropin binding enhancer) binding site.

The use of antigen-antibody complexes precipitation techniques, provided information on differences between normal and Graves' IgG antigenic specificity since two proteins of molecular weights 144,000 and 132,000 were precipitable by Graves' and only minimally by normal IgGs.

The studies described allowed to conclude that the binding site of Graves' IgG is not exactly the same as the one for TSH, since: 1. at least 20% of the patients' IgG tested in the TBI assay did not inhibit thyrotropin binding to the TSH-R, 2. NANA specifically increased the binding of ^{125}I-TSH to the TSH-R, but the modification found in the TBI results of Graves' IgG, in presence of the carbohydrate were non-specific for Graves' IgG, 3. Binding of ^{125}I-TSH to human thyroid cell membrane TSH-R blotted to NC was not inhibited by the presence of Graves' IgG, 4. IgG from patients with Graves' disease was found to bind the different proteins of the human thyroid cell preparations, once these were blotted to NC paper. However, it is still possible that the two TSH binding molecules (128,000 and 80,000 dalton) are components of a receptor unit and that their separation impedes Graves' IgG binding.

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